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14. ABSTRACT

We sought to establish preclinical efficacy of Posiphen as a treatment of traumatic brain injury (TBI). We tested Posiphen in two established animal models of TBI, the Lateral Fluid Percussion (LFP) injury model and the Controlled Cortical Impact (CCI) injury model. Both injury models produced mild TBI as defined by the lack of observed cognitive deficits in the Y maze alternation, the novel object recognition task and the Morris water maze. A 4 weeks treatment with Posiphen (2.5, 5, and 10 mg/kg i.p.) was well tolerated in rats and all doses reversed the loss of tyrosine hydroxylase induced by mild LFP in the striatum of injured rats; the highest dose additionally decreased microglial activation in the substantia nigra ipsilateral to the injury. Contrary to expectation, Posiphen at the doses used did not affect increases in amyloid precursor protein (APP) induced by injury in the hippocampus. The data indicate that Posiphen mitigated neurodegenerative consequences of mild TBI on the nigrostriatal pathway, which is affected in Parkinson's disease, without improving Alzheimer's disease-related biochemical changes.

15. SUBJECT TERMS

Traumatic brain injury, lateral fluid percussion, controlled cortical impact, rat, APP, tau, alpha-synuclein, drug trial

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1. INTRODUCTION

Our objective was to establish preclinical efficacy of Posiphen as a treatment of TBI to provide the foundation for future clinical studies testing efficacy of this small molecule drug to treat TBI patients. The studies funded to date ("base") intended to demonstrate efficacy by testing Posiphen in two established animal models of TBI, the Lateral Fluid Percussion (LFP) injury model (studies in **Task 1)** and the Controlled Cortical Impact (CCI) injury model (studies in **Task 2)**. We sought to determine if Posiphen prevented injury-induced cognitive impairment, neurodegeneration of the dopaminergic nigro-striatal pathway, and increases in amyloid precursor protein (APP), alpha-synuclein, tau and p-tau in brain.

Our Statement of Work includes additional goals to determine the optimal dosing regimen of Posiphen for future clinical testing: is a one-month short-term treatment sufficient to sustain a long-term blockade of the neurodegenerative effects in the TBI models? (**Task 3**); does it require continuous administration to maintain long term beneficial effects? (**Task 4**); how long after the initiation of brain injury can Posiphen be given and still effectively prevent TBI induced cognitive impairment and neurodegeneration? (**Task 5**); can measuring cerebral glucose metabolic rates (CMRglc) be used as a biomarker to determine efficacy of Posiphen in treating TBI? **(Task 6).** These additional tasks were not included in the base funding; therefore, they have not been initiated and are not part of this report.

2. KEYWORDS

Traumatic brain injury, lateral fluid percussion, controlled cortical impact, rat, APP, tau, alpha-synuclein, drug trial, nigro-striatal dopaminergic neurons, tyrosine hydroxylase, rats, striatum, hippocampus, cerebral cortex, substantia nigra.

3. OVERALL PROJECT SUMMARY

Task 1: Efficacy Studies of Posiphen in the LFP injury model

Task 1a: Study Preparation: We synthesized 220 grams of GMP Posiphen to conduct all of the animal studies (Frontage Laboratories). The material was tested for purity and compliance with our GMP specs approved by the FDA.

Task 1b: Determine the optimal dose of Posiphen to test in the chronic TBI models in rat: We treated naïve, male Sprague Dawley rats (2-3 months of age) with Posiphen (0, 10, 20, 30, 40, 50 mg/kg, i.p.) once daily for 21 days. Fresh drug was made daily by an investigator not involved in injections to the animal and the vials were coded. All drug administrations were performed by investigators unaware of dosage. The animals were then euthanized and the brain levels of APP, tau, p-tau and aSyn were measure in cortical and hippocampal tissue by immunoblotting.

Results:

A. Body weight gain in 2-3 month-old rats administered with Posiphen 10-50 mg/kg (i.p.) over 21 days compared to rats administered vehicle.

Rats administered the lowest dose (10 mg/kg) show no slowing of body weight gain compared to rats injected with vehicle. However, a dose-dependent slowing in body weight gain was observed with doses of 20 mg/kg and higher. Only rats administered the highest doses showed a slight decrease compared to the initial weight and a lack of weight gain (**Fig. 1**). PLEASE NOTE THAT ALL FIGURES ARE IN APPENDIX.

Fig. 1: **Body weight of rats administered vehicle or Posiphen i.p. for 21 days**. Mean+/-SEM (n=5 per group) proportion of body weight on each day of treatment compared to day 1 of treatment. Repeated measures two-way ANOVA: treatment

effect F=13.42, p<0.001; day effect F=106.091, p<0.001; interaction of treatment and day F=5.676, p<0.001. Fisher's LSD post-hoc test for groups shown compared to vehicle or Posiphen 10 mg/kg: *p<0.05, ***p<0.001 for days 8-21.

B. Evidence of mild toxicity at the highest doses tested.

In animals receiving doses of 40 and 50 mg/kg, the decrease in body weight was accompanied by discrete signs of toxicity consisting of red staining around the nostrils. The veterinarian consulted indicated that rats did not need to be euthanized prematurely and concluded that the rats showed porphyrin staining (nonspecific sign of stress or illness) and/or epistaxis (nose bleed). These animals also showed diarrhea and bloody stools in the course of the study. At autopsy the animals injected with the highest doses (40 and 50 mg/kg Posiphen) for 21 days showed discolored liver (slightly yellow, fresh-frozen biopsy taken from all rats) and a slight increase in redness of lung tissue in some rats (biopsies were taken but not analyzed).

C. Verification of purity of Posiphen received and used at UCLA by independent laboratory.

Although the mild toxicity of the highest doses of Posiphen was expected based on QR Pharma experience, we wanted to make sure they were not due to any impurity in the batch of Posiphen manufactured for this study. Samples were sent to the laboratory of Dr. Nigel Greig at the NIH, who verified the purity by HPLC. According to Dr. Greig, "The major peak at 5.382 min was in line with Posiphen and accounts for 98.9% of the total area under the curve. Minor peaks are found at both 1.130 and 1.394 min - and these are more polar than Posiphen (the column is reverse phase). Thus, Posiphen purity is high - approximately 98.9%". Therefore, we are confident in the purity of the compound used in the *in vivo* studies.

D. Effects of Posiphen on baseline APP, tau, p-tau, and alpha-synuclein levels.

We had planned to use evidence of effect of Posiphen on levels of APP, tau, P-tau, and/or alpha-synuclein in brain to determine the range of doses to be used for the efficacy studies. Rats were injected with vehicle or 10, 20 or 30 mg/kg Posiphen daily for 21 days. At the end of the treatment period, rats were euthanized by decapitation 3 hours after the last injection of Posiphen. Brains were removed and cut using a coronal brain matrix. Cortical tissue was dissected out from rostral regions (striatum level) and also caudal regions (hippocampus level). Other brain regions harvested included the striatum, olfactory bulb, ventral midbrain, thalamus, cerebellum and medulla. The final group sizes were 5 rats in the vehicle group, 4 rats in the 10 mg/kg group, 5 rats in the 20 mg/kg group, and 4 rats in the 30 mg/kg group. The following antibodies were used: anti-APP (clone 22C11, Millipore, #MAB348); anti-aSyn (BD Biosciences, #610787), anti-tau (Abcam, Ab76128); and anti-phospho-S404-tau (Abcam, Ab92676) (**Fig. 2**). We found that Posiphen did not alter the baseline levels of APP, tau, p-tau, and aSyn in the hippocampus or cortex of uninjured rats (**Fig. 3**).

Fig. 2. **Representative images of immunoblotting for APP, tau, p-tau, and aSyn in brain tissue**. The following antibodies were used: anti-APP at 1:1,000 (clone 22C11, Millipore, #MAB348); anti-aSyn at 1:3,000 (BD Biosciences, #610787), anti-tau at 1:10,000 (Abcam, Ab76128); and anti-phospho-S404-tau at 1:3,000 (Abcam, Ab92676).

Fig. 3. **Posiphen did not alter the levels of APP, tau, p-tau, and aSyn in the hippocampus or cortex of uninjured rats**. Data are shown as the mean +/- S.E.M (vehicle n=5, 10 mg/kg n=4, 20 mg/kg n=5, 30 mg/kg n=4). Significant outliers from each group were removed with the Grubb's test. **(A)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in APP levels between the vehicle and Posiphen-treated groups (Cortex: 80 kDa: F=1.387, p=0.2877; 160 kDa: F=2.920, p=0.0710; total: F=2.841, p=0.0759; Hippocampus: 80 kDa: F=1.448, p=0.2711; 160 kDa: F=1.413, p=0.2805; total: F=1.850, p=0.1845). **(B)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in tau levels between the vehicle and Posiphen-treated groups (Cortex: 50 kDa: F=0.2584, p=0.8541; 55 kDa: F=0.0282, p=0.9933; 60 kDa: F=0.3276, p=0.8054; total: F=0.0877, p=0.9656; Hippocampus: 50 kDa: F=0.9768, p=0.4315; 55 kDa: F=0.6443, p=0.5993; 60 kDa: F=0.3239, p=0.8080; total: F=0.6190, p=0.6142). **(C)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in pS404-Tau levels between the vehicle and Posiphen-treated groups (Cortex: 50 kDa: F=0.1761, p=0.9108; 55 kDa: F=0.1065, p=0.9549; 60 kDa: F=1.608, p=0.2322; total: F=0.3552, p=0.7862; Hippocampus: 50 kDa: F=0.4938, p=0.6924; 55 kDa: F=1.748, p=0.2032; 60 kDa: F=1.033, p=0.4083; total: F=2.279, p=0.1242). **(D)** A one-way ANOVA with Bonferroni

post-hoc comparisons revealed no significant differences in aSyn levels between the vehicle and Posiphen-treated groups (Cortex: F=2.210, p=0.1322; Hippocampus: F=2.410, p=0.1105).

E. Determination of Posiphen levels in the brain.

We injected 3 rats each with 2.5, 5, or 10 mg/kg Posiphen and harvested their brain 30 minutes after drug administration. The levels of Posiphen, N1-Norposiphen and N8-Norposiphen were measured in hippocampal and cortical brain tissue (**Fig. 4**). The samples were analyzed by LC/LC/Mass Spec at Alliance Pharmaceuticals. The 2.5 mg/kg levels are high, but within an acceptable range. As expected from previous PK data with Posiphen, 5 mg/kg i.p. administration of Posiphen results in brain levels of 1000 ng/gram. As expected, levels of N1 and N8 Norposiphen are substantially lower than Posiphen at 30 minutes. From these results, we can conclude that even the lowest dose of 2.5 m/kg i_.p. is an adequate dose to obtain efficacy in the TBI models.

Fig. 4. **Levels of Posiphen, N1-Norposiphen and N8-Norposiphen in hippocampal and cortical brain tissue** (n=3 per group). (A) Posiphen levels, cortical samples p=0.157, hippocampal samples p=0.143, combined samples p= 0.009; (B) N1-Norposiphen levels, cortical samples p=0.004, hippocampal samples p=0.007, combined samples p= 0.001; (C) N8-Norposiphen levels, cortical samples p=0.003, hippocampal samples p=0.004, combined samples p= 0.001; ANOVA.

Discussion:

In the initial phase of the study, we determined that doses of 40 and 50 mg/kg of Posiphen showed adverse effects in rats. These results establish upper limit of doses that cause toxicity in rats. In contrast, doses of 30 mg/kg or lower did not induce overt signs of toxicity *in vivo* or post-mortem, even though doses above 10 mg/kg caused a slight decrease in weight gain.

We did not observe decreases in baseline APP, tau, p-tau, and alpha-synuclein in brain tissue by Western blotting as expected based on previous reports. Therefore, we could not use these data in our determination of the optimal Posiphen dose as originally proposed. However, Dr. Maccecchini previously obtained results showing behavioral benefits without effects on these biochemical markers. Measurements of brain levels of Posiphen, N1-Norposiphen and N8-Norposiphen confirmed that appropriate levels of Posiphen were present in brain. Therefore, in consultation with our program officer, we pursued the study by administering 2.5, 5, and 10 mg/kg Posiphen daily (Task 1c-f and Task 2). ***This strategy proved to be correct as we observed strong protective effects of Posiphen on the nigrostriatal dopaminergic pathway in TBI rats (see below).***

Task 1c. Induce LFP injury and treat with Posiphen: Rats were subjected to a sham operation or LFP injury. The LFP injured (FPI) rats were divided into 4 groups and treated daily with an i.p. injection of vehicle (PBS) or 2.5, 5, or 10 mg/kg Posiphen for 4 weeks.

Death, exclusions and final group sizes:

Based on our approved SOW, each group, including the sham operated (vehicle treated) group originally consisted of 20 rats for a total of 100 rats studied; 120 rats were approved to account for animal loss. Due to loss of rats from death or exclusion, we added 5 rats that were already approved for use in subsequent tasks. Accordingly a total of 125 rats were used for this part of the study, either as sham or subjected to LFP (**Table 1, in appendix**), with a total of 103 rats receiving LFP injury,

A total of 15 rats died during the study period. After review of the surgical records, 9 animals were excluded from subsequent analysis (i.e., suboptimal injury with a short loss of consciousness). In addition, 25 animals were excluded from subsequent analysis based on their poor/lack of performance in the spatial alternations (SA) behavioral test, based on the protocol established by the Hovda laboratory for these cognitive tests. The

exclusion criteria consisted of a pre-treatment SA score of less than 57% or less than 11 total arm entries (e.g., those rats did not complete the test).

The final groups for analyses consisted of: 16 rats in the FPI/Vehicle group, 16 rats in the FPI/2.5 mg/kg Posiphen group, 15 rats in the FPI/5.0 mg/kg Posiphen group, 14 rats in the FPI/10 mg/kg Posiphen group, and 15 rats in the Sham FPI/Vehicle group. As described in our project narrative, these final group sizes were fully powered to detect behavioral, biochemical, and histological differences (Tasks 1d-f). Indeed, previous studies [1] showed a significant group effect for latency reaching the hidden platform ($p < 0.05$) in the Morris Water Maze (MWM) in LFP injured rats 21 days after injury with as few as 8 animals per group. Furthermore, significant ($p<0.05$) changes in stereological endpoints of nigrostriatal dopaminergic neurons and microglial activation after LFP were seen with 6-8 animals [2].

As previously described [2], we used the duration of loss of consciousness (LOC) to determine that magnitude of injury, with a target around 200 seconds to obtain a mild TBI, without visible histological lesion in the cerebral cortex. All animals included in the analyses had a LOC between 105 and 450 seconds (with the majority of animals between 105 and 300). The mean LOC for the FPI rats included in the final analysis, after exclusions, is shown in **Fig. 5**. There was no significant difference in mean LOC among groups. We subsequently analyzed all data in relation to the LOC observed in individual rats. We generated correlation analyses and also analyzed separately data obtained in rats with LOC above and below the group median. We did not observe any positive or negative correlation between LOCs and behavioral, histological or biochemical data reported here. Subgroup analyses did not uncover more significant changes when only animals with the longest LOC were included. Therefore, all subsequent data are reported as means per group.

Fig. 5. **Mean loss of consciousness (LOC) for the FPI groups**. Data are shown as the mean +/- S.E.M (FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=16, FPI/5.0 mg/kg Posiphen n=15, FPI/10 mg/kg Posiphen n=14). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between groups ($F=1.375$, $p=0.2597$).

Discussion:

The very large majority of animals in this study (116 out of 125) presented with a LOC in the range expected based on the protocol in routine use in the Hovda laboratory to induce mild to moderate TBI by LFP injury. The number of death was compatible with prior experience in the Hovda laboratory (15 out of 125 rats). The largest number of rats removed form the study (25) was based on the exclusion protocol used in the Hovda laboratory to select rats able to perform the cognitive task by testing them in the Y maze before surgery. Nevertheless, all groups included 14-16 rats at the end of the study, which is within the expected range to detect effects based on power analyses performed before the study was initiated. The resulting groups of rats had consistent LOC in the range expected to produce mild TBI and our in depth analyses indicate that there is no variability in the data within this range of injury severity. In other words, we are confident that no effect is "missed" by including animals with the full range of LOC durations in the final groups.

Task 1d: Cognitive tests (in animals described in task 1c):

Optimization of cognitive tasks proposed in the SOW:

Prior to performing cognitive testing in our efficacy study, we conducted pilot studies to optimize the tasks. Even though the Hovda laboratory had previously reported deficits in these tasks after mild TBI, we found that the reference memory during the Morris Water Maze (MWM) was not affected under our experimental conditions after TBI; however, we detected deficits in the working memory version of the task after TBI and subsequently used this version (described below).

We also performed several versions of the Novel Object Recognition (NOR) task, with little evidence of a high discrimination index in naïve rats. After consultation with colleagues in the Behavioral Core facility, we slightly

modified the conditions and obtained an acceptable discrimination index in naïve rats; however, this test showed no deficits in FPI rats 13 days after surgery, suggesting that our experimental conditions resulted in mild TBI without cognitive deficits. We included the NOR test in our study as planned with the following modifications: (1) a longer delay (40 minutes) between exposure to the initial two objects and exposure to the novel object and (2) performing the test at an earlier time after surgery (described below).

Statistical analyses:

Groups of sham and vehicle-injected FPI rats were first compared with a Student's t test to determine whether or not the LFP injury induced a cognitive deficit. Additionally, we compared the 4 groups of FPI rats (vehicle, 2.5 mg/kg, 5mg/kg and 10 mg/kg) with an ANOVA followed by post-hoc analyses with Bonferroni corrections to ensure that Posiphen did not induce any cognitive deficits in FPI rats. PLEASE NOTE THAT ONLY SIGNIFICANT DIFFERENCES BETWEEN SHAM AND VEHICLE (*) and BETWEEN EACH POSIPHEN DOSE COMPARED TO THE VEHICLE LFP GROUP (#) ARE INDICATED ON THE GRAPHS (i.e. no symbols are included to indicate differences between sham and Posiphen groups).

Results:

A. No effect of LFP injury on spatial alternations, and no detrimental effects of Posiphen.

Rats were placed in the middle of the maze and left to explore for 15 minutes. The number and sequence of arm entries were recorded for calculation of a percent alternation score. An alternation consists of 4 different arm choices of 5 consecutive arm entries. A 4/5 percent alternation score was computed by dividing the number of observed alternations in overlapping quintuplets by the number of possible alternations and multiplying the quotient by 100 (**Fig. 6**). Rats underwent pre-surgery spatial alternation (SA) testing 4 days prior to LFP or sham surgery, and again at post-operative day (POD) 16 and 29 (**Fig. 7**).

Fig. 6. **Image of the maze used for the spatial alternation task**. Rats were placed in the middle of the maze and left to explore for 15 minutes. The number and sequence of arm entries were recorded for calculation of a percent alternation score. An alternation consists of 4 different arm choices of 5 consecutive arm entries. A 4/5 percent alternation score was computed by dividing the number of observed alternations in overlapping quintuplets by the number of possible alternations and multiplying the quotient by 100.

Fig. 7. **No effect of LFP injury on spatial alternations**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=16, FPI/5.0 mg/kg Posiphen n=15, FPI/10 mg/kg Posiphen n=14). A two-way repeated-measures ANOVA with Bonferroni post-hoc comparisons revealed a significant effect of time ($F=5.319$, $p=0.0062$), but no significant differences between the vehicle and Posiphen-treated groups ($F=0.2866$, $p=0.8349$). Planned comparisons (sham vs. vehicle) within each time point also revealed no significant differences. POD = post-operative day.

B. No effect of LFP injury on novel object recognition, and no detrimental effect of Posiphen.

Rats underwent NOR testing on days 9, 10, and 11 after LFP or sham injury (**Fig. 8**). The first 2 days were habituation days, in which the rats were habituated to the tank (5 min per rat). Day 3 was the testing day, which consisted of 2 trials. Trial 1 was the Familiarization test, consisting of 10 min of exploration time per rat and 2 identical objects (plastic yellow eggs or small plastic orange basketballs). The rats were then given a 40 minutes inter-trial interval. Trial 2 consisted of the memory testing. Here, one object was replaced with a novel object and the rat was given 10 minutes to explore. The discrimination index (DI) was calculated using novel (N) and familiar (F) sniffing times with the formula: N-F/N+F.

Fig. 8. **No effect of LFP injury on novel object recognition.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=15, FPI/5.0 mg/kg Posiphen n=14, FPI/10 mg/kg Posiphen n=13). A Student's t-test between sham and vehicle groups revealed no significant differences ($t=0.3645$, $p=0.7181$). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-

treated groups (F=0.9716, p=0.4130). Note that data from 1 animal each from the 2.5, 5, and 10 mg/kg groups were not used because both objects were not explored during testing. DI = discrimination index.

C. No effect of LFP injury on working memory in the Morris water maze, and no detrimental effect of Posiphen.

For a total of 3 days, rats were tested on their ability to find 4 platform positions, using paired trials (8 total trials per day). The platform positions (1, 2 3 and 4) were switched for every trial. N, S, E and W were the release points (**Fig. 9**). Rats were allowed a maximum swim time of 90 seconds (guided to platform if needed after 90 sec). Trial 2 (T2) was started 5 seconds later. The time between Trial 1 (T1) at each new platform position was 5 min. The percent improvement in finding the platform was calculated using the following formula: 1 – T2/T1 * 100%. Rats underwent MWM testing on days 22, 23, and 24 following LFP or sham injury (**Fig. 10**).

Fig. 9. **Diagram of the platform positions and release points used in the Morris water maze**. For a total of 3 days, rats were tested on their ability to find 4 platform positions, using paired trials (8 total trials per day). The platform positions (1, 2 3 and 4) were switched for every trial. N, S, E and W were the release points (Fig. Rats were allowed a maximum swim time of 90 seconds (guided to platform if needed after 90 sec). Trial 2 (T2) was started 5 seconds later. The time between Trial 1 (T1) at each new platform position was 5 min. The percent improvement in finding the platform was calculated using the following formula: 1 – T2/T1 * 100%.

Fig. 10. **No effect of LFP injury on working memory in the Morris water maze**. Averaged data from the 3 testing days are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=15, FPI/5.0 mg/kg Posiphen n=14, FPI/10 mg/kg Posiphen n=13). A Student's t-test between sham and vehicle groups revealed no significant differences (t=1.445, p=0.1592). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.8984, p=0.4481). Note that data from 1 animal each from the 2.5, 5, and 10 mg/kg groups were not used because of a technical error during testing.

Discussion:

Extensive testing using three different cognitive tests (Y maze alternation, Novel Object Recognition, and the working memory version of the MWM) did not detect any cognitive deficits in the rats having undergone LFP in this study. This was unexpected based on previous data from the Hovda lab and our pilot study. We do not have a definitive explanation for this observation, except that daily handling and injection stress may have interfered with our ability to detect injury-induced deficits. It should also be noted that our goal was to induce a mild TBI, which clinically is defined by the absence of behavioral deficit.

Even though the lack of LFP-induced deficits prevented us from determining whether Posiphen has a positive functional effect after TBI, two important findings resulted from this study:

1. Posiphen did not induce cognitive deficits even in rats with brain function compromised by LFP, reinforcing the notion that the drug is safe.

2. As will be seen below, our post-mortem data confirm that even in the absence of behavioral deficits, LFP has histological and biochemical consequences that are associated with neurodegenerative diseases and may represent early deficits which can lead to later neurodegeneration.

Task 1e: Test for Neurochemical changes in brain after LFP injury:

As described in our SOW, we separated each group in half to allow for both histological and neurochemical post-mortem analyses.

Groups, Euthanasia, and Tissue preparation:

Half of the rats subjected to LFP or sham injury were randomly assigned at the onset of the study (to avoid any bias) to the neurochemistry groups and their brain fresh-frozen and processed for Western blotting to assess changes in APP, tau, p-tau, BDNF, and aSyn. After exclusion of rats for death, LOC or low pre-injury low cognitive performance (see task 1c for criteria), the final group sizes were: 7 rats in the Sham FPI/Vehicle group, 9 rats in the FPI/Vehicle group (8 for hippocampal tissue due one lost sample), 7 rats in the FPI/2.5 mg/kg Posiphen group, 6 rats in the FPI/5.0 mg/kg Posiphen group, and 6 rats in the FPI/10 mg/kg Posiphen group.

The rats were deeply anesthetized, and we obtained blood using cardiac puncture. Serum, plasma, and red blood cell preparations were frozen on dry ice and stored at -80°C. Rats were transcardially perfused with saline (this is required for measuring alpha-synuclein, which is abundant in blood), and brains were removed immediately. The right and left hippocampi, along with overlying cortical regions were dissected on ice. These regions, along with the remainder of the brain, and lung and liver samples were snap-frozen in liquid nitrogen and stored at -80°C. Tissue from the cortex and hippocampus (ipsilateral to the lesion) was processed and subjected to SDS-PAGE. The following antibodies were used for the subsequent immunoblotting: anti-APP (clone 22C11, Millipore, #MAB348); anti-aSyn (BD Biosciences, #610787); anti-tau (Abcam, Ab76128); anti-phospho-S404-tau (Abcam, Ab92676); and anti-BDNF (Abcam, Ab109049). B-actin was used as a loading control (Abcam, Ab6276).

Statistical analyses:

Groups of sham and vehicle-injected FPI rats were first compared with a Student's t test to determine whether or not the LFP injury induced a change in protein levels. Additionally, we compared the 4 groups of FPI rats (vehicle, 2.5 mg/kg, 5mg/kg and 10 mg/kg) with an ANOVA followed by post-hoc analyses with Bonferroni corrections to determine whether Posiphen induced any changes compared to vehicle-treated FPI animals. PLEASE NOTE THAT ONLY SIGNIFICANT DIFFERENCES BETWEEN SHAM AND VEHICLE (*) and BETWEEN EACH POSIPHEN DOSE COMPARED TO THE VEHICLE LFP GROUP (#) ARE INDICATED ON THE GRAPHS (i.e. no symbols are included to indicate differences between sham and Posiphen groups).

Results:

A. LFP injury significantly increased APP levels in the hippocampus; no effect of Posiphen (**Fig. 11**).

Fig. 11. **LFP injury significantly increased APP levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed a significant increase in the 80 kDa band of APP and total APP levels in injured rats (80 kDa: t=2.222, *p=0.0446; 160 kDa: t=1.444, p=0.1723; total: t=2.533, *p=0.0250). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.6501, p=0.5909; 160 kDa: F=0.1132, p=0.9515; total: F=0.6569, p=0.5869). Significant outliers from each group were removed with the Grubb's test.

B. LFP injury did not significantly affect aSyn levels in the hippocampus; no effect of Posiphen (**Fig. 12**).

Fig. 12. **LFP injury did not significantly affect aSyn levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=1.173, p=0.2618). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.658, p=0.2038). Significant outliers from each group were removed with the Grubb's test.

C. LFP injury did not significantly affect BDNF levels in the hippocampus; no effect of Posiphen (**Fig. 13**).

Fig. 13. **LFP injury did not significantly affect BDNF levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=1.579, p=0.1402). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.205, p=0.3313). Significant outliers from each group were removed with the Grubb's test.

D. LFP injury did not significantly affect Tau levels in the hippocampus; no effect of Posiphen (**Fig. 14**).

Fig. 14. **LFP injury did not significantly affect Tau levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=0.1708, p=0.8670; 55 kDa: t=0.6774, p=0.5100; 60 kDa: t=0.9264, p=0.3711; total: t=0.5609, p=0.5844). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.6467, p=0.5933; 55 kDa: F=1.017, p=0.4050; 60 kDa: F=0.6257, p=0.6064; total: F=0.6232, p=0.6079). Significant outliers from each group were removed with the Grubb's test.

E. LFP injury induced a strong trend towards increased pTau-S404 50 kDa levels in the hippocampus; no effect of Posiphen (**Fig. 15**).

Fig. 15. **LFP injury showed a strong trend towards increased pTau-S404 levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=5-6). A Student's t-test between sham and vehicle groups revealed a strong trend toward a significant increase in the pS404-tau 50 and 55 kDa bands in injured rats (50 kDa: t=2.116, p=0.0542; 55 kDa: t=1.922, p=0.0768; 60 kDa: t=1.350, p=0.2000; total: t=1.810, p=0.0935). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.489, p=0.2450; 55 kDa: F=0.2879, p=0.8336; 60 kDa: F=0.3299, p=0.8037; total: F=0.4063, p=0.7499). Significant outliers from each group were removed with the Grubb's test.

F. LFP injury did not significantly affect the pTau/tau ratio in the hippocampus (**Fig. 16**).

Fig. 16. **LFP injury did not significantly affect the pTau/tau ratio in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=4-5). A Student's t-test between sham and vehicle groups revealed no significant differences in the ratio of pTau-S404/tau (50 kDa: t=0.8417, p=0.4152; 55 kDa: t=0.4350, p=0.6707; 60 kDa: t=0.4995, p=0.6258; total: t=0.6208, p=0.5455). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.011, p=0.4076; 55 kDa: F=1.007, p=0.4092; 60 kDa: F=0.3787, p=0.7694; total: F=0.7955, p=0.5101). Significant outliers from each group were removed with the Grubb's test.

G. LFP injury did not significantly affect APP levels in the cerebral cortex (**Fig. 17**).

Fig. 17. **LFP injury did not significantly affect APP levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8-9, FPI/2.5 mg/kg Posiphen n=6-7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences (80 kDa: t=0.4624, p=0.6509; 160 kDa: t=0.5134, p=0.6720; total: t=0.3077, p=0.7628). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.8791, p=0.4658; 160 kDa: F=0.8770, p=0.4681; total: F=1.346, p=0.2841). Significant outliers from each group were removed with the Grubb's test.

H. LFP injury did not significantly affect aSyn levels in the cerebral cortex (**Fig. 18**).

Fig. 18. **LFP injury did not significantly affect aSyn levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=5, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=1.662,

p=0.1204). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.940, p=0.1526). Significant outliers from each group were removed with the Grubb's test.

I. LFP injury did not significantly affect BDNF in the cerebral cortex (**Fig. 19**).

Fig. 19. **LFP injury did not significantly affect BDNF in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6, FPI/vehicle n=9, FPI/2.5 mg/kg Posiphen n=6, FPI/5.0 mg/kg Posiphen n=5, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=0.7770, p=0.4511). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.923, p=0.1553). Significant outliers from each group were removed with the Grubb's test.

J. LFP injury significantly decreased Tau 60 kDa levels in the cerebral cortex; no effect of Posiphen (**Fig. 20**).

Fig. 20. **LFP injury significantly decreased Tau levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6-7, FPI/vehicle n=8-9, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups a significant decrease in the 60 kDa tau band in injured rats (50 kDa: t=1.297, p=0.2157; 55 kDa: t=1.387, p=0.1872; 60 kDa: t=2.259, *p=0.0433; total: t=1.440, p=0.1719). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.3838, p=0.7656; 55 kDa: F=0.2250, p=0.8781; 60 kDa: F=0.6425, p=0.5955; total: F=0.2697, p=0.8466). Significant outliers from each group were removed with the Grubb's test.

K. LFP injury did not significantly affect pTau-S404 levels in the cerebral cortex (**Fig. 21**).

Fig. 21. **LFP injury did not significantly affect pTau-S404 levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in pTau-S404 levels (50 kDa: t=0.1943, p=0.8487; 55 kDa: t=0.0619, p=0.9515; 60 kDa: t=0.6874, p=0.5031; total: t=0.2330, p=0.8191). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.7531, p=0.5318; 55 kDa: F=0.5570, p=0.6487; 60 kDa: F=0.7203, p=0.5501; total: F=0.6714, p=0.5783). Significant outliers from each group were removed with the Grubb's test.

L. LFP injury does not significantly affect the pTau/tau ratio in the cerebral cortex (**Fig. 22**).

Fig. 22. **LFP injury did not significantly affect the pTau/tau ratio in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=7-8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=4). A Student's t-test between sham and vehicle groups revealed no significant differences in the pTau-S404/tau ratio (50 kDa: t=1.151, p=0.2706; 55 kDa: t=1.743, p=0.1049; 60 kDa: t=1.457, p=0.1708; total: t=1.391, p=0.1877). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.5130, p=0.6777; 55 kDa: F=0.0411, p=0.9886; 60 kDa: F=0.3415, p=0.7955; total: F=0.1697, p=0.9156). Significant outliers from each group were removed with the Grubb's test.

Discussion:

Our results confirmed that even mild TBI induced by LFP increases levels of APP (80 kDa band and total levels) a protein linked to Alzheimer's disease pathology, in the hippocampus. However, contrary to expectation, Posiphen did not attenuate this effect. As indicated earlier, brain levels of Posiphen were verified in this study and found to correspond to the range known to exert its effects. Furthermore, higher doses of Posiphen induced significant toxicity. Therefore, the lack of effect on APP levels was <u>not</u> due to the use of a suboptimal dose of the drug.

Other biochemical parameters were not significantly affected in vehicle-injected FPI rats compared to shams. It should be noted that all data are presented in rats retained after application of exclusion criteria for low LOC and inability to perform the cognitive tasks prior to TBI injury for consistency across all measurements. However, we verified that including the excluded animals, for a total of n=10 per group, did not reveal any changes in tau, ptau, ptau/tau ratio, alpha-synuclein or BDNF after LFP either. Therefore, the lack of effects observed in this cohort is not due to the small group of animals used for final analysis. Similarly, a separate analysis of animals with the longest LOC during LFP did not uncover additional significant biochemical changes. Therefore, those changes were not present even in the subgroup of rats with the most severe injury.

Task 1f: Test for Neurodegeneration of nigro-striatal DA neurons after LFP injury:

Groups, Euthanasia, and Tissue preparation:

The brains from half of the rats subjected to LFP or sham injury were used to assess nigrostriatal DA neuron degeneration, microglial activation, and striatal tyrosine hydroxylase (TH) immunoreactivity. Based on the exclusion criteria described in Task 1c, the final group sizes were: 8 rats in the Sham FPI/Vehicle group, 7 rats in the FPI/Vehicle group, 9 rats in the FPI/2.5 mg/kg Posiphen group, 9 rats in the FPI/5.0 mg/kg Posiphen group, 8 rats in the FPI/10 mg/kg Posiphen group.

The rats were deeply anesthetized and we obtained blood from the rats using cardiac puncture. Serum, plasma, and red blood cell preparations were frozen on dry ice and stored at -80°C. Rats were then transcardially perfused with PBS and we obtained lung and liver tissue. This was followed by transcardial perfusion with 4% PFA. The brains were removed, post-fixed overnight in PFA, immersed in sucrose for cryoprotection, frozen in crushed dry ice, and stored at -80°C.

Tyrosine hydroxylase (TH) immunoreactivity, a marker of nigrostriatal axon terminals, was detected by immunofluorescence staining in sections of the rostral, medial, and caudal striatum ipsilateral to side of injury and quantified using a microarray scanner [4]. The numbers of TH positive neurons (corresponding to dopaminergic cell bodies) and of total neurons (Nissl+) were estimated using stereological methods in the substantia nigra ipsilateral to side of injury. Microglial activation in substantia nigra was quantified by measuring the soma diameter of IBA-1+ cells ipsilateral to the side of injury as previously described by our laboratory [5]. The antibodies used included anti-TH (PelFreez, #P40101-0) and anti-IBA-1 (Wako, #019-19741).

Statistical analyses:

For TH immunostaining in the striatum and for cell numbers in the substantia nigra, groups of sham and vehicle-injected FPI rats were first compared with a Student's t test to determine whether or not the LFP injury induced a change. Additionally, we compared the 4 groups of FPI rats (vehicle, 2.5 mg/kg, 5mg/kg and 10 mg/kg) with an ANOVA followed by post-hoc analyses with Bonferroni corrections to determine whether Posiphen induced any changes compared to vehicle-treated FPI animals. PLEASE NOTE THAT ONLY SIGNIFICANT DIFFERENCES BETWEEN SHAM AND VEHICLE (*) and BETWEEN EACH POSIPHEN DOSE COMPARED TO THE VEHICLE LFP GROUP (#) ARE INDICATED ON THE GRAPHS (i.e. no symbols are included to indicate differences between sham and Posiphen groups).

Distributions of the soma sizes of IBA1 cells were compared with a bootstrapping statistic as previously described [5]. On the graphs, each vertical bar represents the percent of cells with a given diameter, with the colored portion of the bar representing the confidence interval. A statistical difference between the groups is identified by non-overlapping confidence intervals.

Results:

A. FPI-induced a marked decrease in TH+ terminals in the ipsilateral striatum; this injury effect was reversed by all doses of Posiphen (**Fig. 23**).

Fig. 23. **Treatment with Posiphen significantly attenuated the FPI-induced decrease in TH+ terminals in the ipsilateral striatum.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=8, FPI/vehicle n=6, FPI/2.5 mg/kg Posiphen n=8, FPI/5.0 mg/kg Posiphen n=8, FPI/10 mg/kg Posiphen n=7). **(A)** A Student's t-test between sham and vehicle groups revealed a significant decrease in TH-ir in the striatum (t=2.470, *p=0.0295). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in all Posiphen-treated groups when compared with the vehicle (F=5.499, #p<0.05 when compared with vehicle). **(B)** A Student's t-test between sham and vehicle groups revealed a strong trend toward a decrease in TH-ir in the rostral striatum (t=1.960, p=0.0736). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 10 mg/kg Posiphen when compared with the vehicle (F=3.310, #p<0.05 when compared with vehicle). **(C)** A Student's t-test between sham and vehicle groups revealed no significant differences in TH-ir in the medial striatum (t=1.756, p=0.1045). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 2.5 and 5 mg/kg Posiphen when compared with the vehicle (F=4.605, #p<0.05 when compared with vehicle). **(D)** A Student's t-test between sham and vehicle groups revealed a significant decrease in TH-ir in the caudal striatum (t=2.667, *p=0.0205). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 2.5 and 5 mg/kg Posiphen when compared with the vehicle (F=4.414, #p<0.05 when compared with vehicle). Significant outliers from each group were removed with the Grubb's test.

B. LFP injury did not significantly affect the number of TH+ or Nissl+ neurons in the ipsilateral substantia nigra (**Fig. 24**, **Table 2, Fig. 25**).

Fig. 24. **Representative image of brain tissue processed for TH-immunoreactivity with a Nissl counterstain**. Subregions of the substantia nigra as defined by the "The Rat Brain Atlas (Paxinos 4th Edition)" are marked with white text. The image was taken at 5X (Scale bar = 200 um). The "dorsal" and medial regions correspond to substantia nigra pars compacta, which is affected in Parkinson's disease. The ventral region correspond to dopaminergic cell bodies scattered within the pars reticulata that project to the striatum as well and are considered by some to be the ventral portion of the pars compacta.

Although we did not find any significant effect of the TBI on the number of TH+ in any subregion of the substantia nigra **(Table 2)**, we noticed a trend towards a (non significant) decreased mean number of TH+ neurons in the ventral group. A very strong trend (Student's t test, one tailed p=0.059) was observed when data were analyzed with a one tailed t test, which is justified since we only expect a decrease in cell numbers **(Fig. 25)**. This effect was not seen on the number of Nissl-stained neurons, indicating that it is due to a decrease in TH staining not cell death.

Table 2. **LFP injury did not significantly affect the number of TH+ or Nissl+ neurons in the ipsilateral substantia nigra**. Groups include: Sham FPI/Vehicle (n=8), FPI/Vehicle (n=7), FPI/2.5 mg/kg Posiphen (n=9), FPI/5.0 mg/kg Posiphen (n=9), FPI/10 mg/kg Posiphen (n=6-7). Significant outliers from each group were removed with the Grubb's test.

Fig. 25. **Scatter plot of the cell estimates for TH+ neurons (A) and the TH+/Nissl+ neurons (B) in the ventral SN**. Groups include: Sham FPI/Vehicle (n=8), FPI/Vehicle (n=7), FPI/2.5 mg/kg Posiphen (n=9), FPI/5.0 mg/kg Posiphen (n=9), FPI/10 mg/kg Posiphen (n=7). Significant outliers from each group were removed with the Grubb's test.

C. FPI increased microglial activation in the ipsilateral substantia nigra, an effect attenuated by 10mg/kg of Posiphen (**Fig. 26**).

Fig. 26. Treatment with 10 mg/kg Posiphen significantly attenuates the FPI-induced increase in microglial activation in the ipsilateral substantia nigra. Data (Mean + 95% CI) were analyzed with Bootstrapping method, *p<0.05 (Sham FPI/vehicle n=8, FPI/vehicle n=7, FPI/2.5 mg/kg Posiphen n=9, FPI/5.0 mg/kg Posiphen n=9, FPI/10 mg/kg Posiphen n=7). **(A)** FPI rats showed a significant increase in microglial activation when compared with the vehicle control. **(B)** Treatment with 2.5 mg/kg Posiphen showed no effect on microglial activation when compared with the vehicle control. **(C)** Treatment with 5.0 mg/kg Posiphen showed no effect on microglial activation when compared with the vehicle control. **(D)** Treatment with 10.0 mg/kg Posiphen showed a slight decrease in microglial activation when compared with the vehicle control.

Discussion

Our histological analysis revealed that even mild LFP-induced TBI damages the nigrostriatal pathway as evidenced by a decrease in the level of tyrosine hydroxylase in the terminals of nigrostriatal dopaminergic neurons. The effect was observed throughout the striatum, reaching significance overall and in the caudal region. This is of particular interest because the causal striatum in rats is the equivalent of the putamen in primates, the region first affected in the course of nigrostriatal degeneration in Parkinson's disease. Posiphen reversed this deficit, indicating a beneficial effect of the drug in brain even at the lowest concentration used in this study.

We observed a trend towards a reduction of the number of dopaminergic cell bodies in the ventral part of the substantia nigra, a region projecting to the caudal striatum where the effect on terminals was the strongest. The trend was clearly reversed in animals that received Posiphen. This supports the notion that TBI may affect dopaminergic cell bodies as well as their terminals, as shown by low tyrosine hydroxylase levels in this subpopulation of dopaminergic neurons, but as we have previously shown [2], longer time post-injury may be necessary to observe a significant decrease. Again, it is important to stress the experimental differences with our previous studies, which were performed in rats that were not handled after the initial LFP, in contrast to this study in which animals were handled and injected daily i.p..

The mechanism by which Posiphen protects against TBI-induced damage to the nigrostriatal neurons, after LFP is unclear. Posiphen improved microglial activation in the substantia nigra but this effect was only seen with the highest dose, suggesting that it is not causally related to the improvement of tyrosine hydroxylase levels in the striatum, which was observed after all doses used in this study. Future studies of alpha-synuclein expression, a protein linked to Parkinson's disease pathology that was previously found increased after TBI, would be of interest.

Task 2: Efficacy Studies in the CCI model

Task 2a. Induce CCI injury and treat with Posiphen:

Rats were subjected to a sham operation or CCI injury. The CCI injured rats were divided into 4 groups and treated daily with an i.p. injection of vehicle (PBS) or 2.5, 5, or 10 mg/kg Posiphen for 4 weeks. A total of 66 rats were originally assigned to this portion of the study. A total of 4 rats died during the course of the study period. The routine procedure for evaluating injury severity after CCI is to examine the cortex post mortem for evidence of swelling. Based on these observations, 7 animals were excluded from subsequent analysis due to suboptimal injury with limited cortical swelling. Lastly, 8 animals were excluded from subsequent analysis based on their inability to satisfactorily perform the cognitive task in spatial alternations testing (SA) prior to surgery. Similar to the LFP group, these exclusion criteria consisted of a pre-treatment SA score of less than 57% or less than 11 total arm entries (e.g., the animal did not complete the test).

Accordingly, the final sizes of groups for analyses were: 10 rats in the Sham CCI/Vehicle group, 10 rats in the CCI/Vehicle group, 10 rats in the CCI/2.5 mg/kg Posiphen group, 8 rats in the CCI/5.0 mg/kg Posiphen group, 9 rats in the CCI/10 mg/kg Posiphen group (**Table 3**). As indicated in the project narrative, these final group sizes were fully powered to detect behavioral, biochemical, and histological differences (Tasks 2b-c). Studies by Moro et al. [3] showed that groups of 7 animals each were sufficient to show significant impairment in cognition in the plus maze in the CCI model compared to sham injured rats. The mean level of cortical swelling for the CCI groups is shown in **Fig. 27**.

Fig. 27. Mean level of cortical swelling for the CCI groups. A qualitative injury scale was used to assess brain swelling: 0.5 = mild-moderate; 1 = moderate; 2 = moderate-severe; 3 = severe. Data are shown as the mean +/- S.E.M. (CCI/vehicle

n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A non-parametric one-way ANOVA (Kruskal-Wallis) revealed no significant differences between groups (p=0.4930).

Task 2b: Cognitive Tests:

The same testing procedures used in Task 1d were implemented. Rats underwent pre-surgery spatial alternation (SA) testing 4 days prior to CCI or sham surgery, and again at post-operative day (POD) 16 and 29. Rats underwent MWM testing on days 22, 23, and 24 following CCI or sham injury. Rats underwent NOR testing on days 9, 10, and 11 after CCI or sham injury.

Results:

A. No effect of CCI injury on spatial alternations; no Posiphen effects (**Fig. 28**).

Fig. 28. **No effect of CCI injury on spatial alternations**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A two-way repeated-measures ANOVA with Bonferroni post-hoc comparisons revealed a significant effect of time (F=5.801, p=0.0048), but no significant differences between the vehicle and Posiphen-treated groups (F=0.4714, p=0.7043). Planned comparisons (sham vs. vehicle) within each time point also revealed no significant differences. POD = post-operative day.

B. No effect of CCI injury on novel object recognition; no Posiphen effects (**Fig. 29**).

Fig. 29. **No effect of CCI injury on novel object recognition**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences (t=0.08391, p=0.9341). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.3039, p=0.8223). Note that data from 1 animal each from the vehicle, 2.5, and 10 mg/kg groups were not used because both objects were not explored during testing. DI = discrimination index.

C. No effect of CCI injury on working memory in the Morris water maze; no Posiphen effects (**Fig. 30**).

Fig. 30. **No effect of CCI injury on working memory in the Morris water maze**. Averaged data from the 3 testing days are shown as the mean +/- S.E.M (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=6, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences (t=0.9135, p=0.3731). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.9733, p=0.4183). Note that data from 2 animals from the 5 mg/kg group and 1 animal from the 10 mg/kg groups were not used because of a technical error during testing.

Discussion:

Similar to LFP in this study, CCI did not induced detectable cognitive deficits despite using routine protocols by an experienced team in the Hovda laboratory. The lesions performed in this study induced the expected level of cortical swelling, similar to that observed in the group's previous studies. As previously discussed, daily handling and the stress of i.p. injections, may have interfered with our ability to detect subtle cognitive deficits. Nevertheless, this result is compatible with our goal of inducing mild TBI to focus on long-term TBI-induced brain damage. Furthermore, results in this cohort confirmed that Posiphen did not induce any unwanted cognitive deficits, even in animals with a brain compromised by a mild TBI.

Task 2c: Test for Neurochemical changes in brain after CCI injury:

No histological analysis was proposed for rats that underwent injury by CCI. Therefore, the brains from all rats subjected to CCI or sham injury were used to assess changes in APP, tau, p-tau, BDNF, and aSyn by quantitative immunoblotting.

The rats were deeply anesthetized and we obtained blood from the rats using cardiac puncture prior to perfusion with saline. Serum, plasma, and red blood cell preparations were frozen on dry ice and stored at -80°C. Rats were then transcardially perfused with saline, and brains were removed immediately. The right and left hippocampi, along with overlying cortical regions were dissected on ice. These regions, along with the remainder of the brain, and lung and liver samples were snap-frozen in liquid nitrogen and stored at -80°C. Tissue from the cortex and hippocampus (ipsilateral to the lesion) was processed and subjected to SDS-PAGE. The following antibodies were used for the subsequent immunoblotting: anti-APP (clone 22C11, Millipore, #MAB348); anti-aSyn (BD Biosciences, #610787); anti-tau (Abcam, Ab76128); anti-phospho-S404-tau (Abcam, Ab92676); and anti-BDNF (Abcam, Ab109049). B-actin was used as a loading control (Abcam, Ab6276).

Results:

A. CCI injury did not significantly affect APP levels in the hippocampus; no effects of Posiphen (**Fig. 31**).

Fig. 31. **CCI injury did not significantly affect APP levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in APP (80 kDa: t=0.2434, p=0.8105; 160 kDa: t=0.7861, p=0.4420; total: t=0.8989, p=0.3806). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.2025, p=0.8939; 160 kDa: F=0.4745, p=0.7022; total: F=0.8299, p=0.4873). Significant outliers from each group were removed with the Grubb's test.

B. CCI injury did not significantly affect aSyn levels in the hippocampus; no effects of Posiphen (**Fig. 32**).

Fig. 32. **CCI injury did not significantly affect aSyn levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=0.5520, p=0.5877). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.7523, p=0.5291). Significant outliers from each group were removed with the Grubb's test.

C. CCI injury did not significantly affect BDNF levels in the hippocampus; no effects of Posiphen (**Fig. 33**).

Fig. 33. **CCI injury did not significantly affect BDNF levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9, CCI/vehicle n=8, CCI/2.5 mg/kg Posiphen n=7, CCI /5.0 mg/kg Posiphen n=5, CCI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=0.1478, p=0.8845). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.0988, p=0.9599). Significant outliers from each group were removed with the Grubb's test. Some animals were excluded due to a lost sample.

D. CCI injury did not significantly affect Tau levels in the hippocampus; no effect of Posiphen (**Fig. 34**).

Fig. 34. **CCI injury did not significantly affect Tau levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=8-9). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=0.1404, p=0.8899; 55 kDa: t=0.1431, p=0.8878; 60 kDa: t=0.2014, p=0.8426; total: t=0.1659, p=0.8701). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.6829, p=0.5692; 55 kDa: F=0.3949, p=0.7575; 60 kDa: F=0.3721, p=0.7737; total: F=0.4062, p=0.7496). Significant outliers from each group were removed with the Grubb's test.

E. CCI injury significantly decreased pTau-S404 (55 kDa) levels in the hippocampus, an effect not reversed by Posiphen (**Fig. 35**).

Fig. 35. **CCI injury significantly decreased pTau-S404 levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed a significant decrease in the 55, 60 kDa, and total pS404-tau bands in injured rats (50 kDa: t=1.438, p=0.1685; 55 kDa: t=2.347, *p=0.0313; 60 kDa: t=d no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.253, p=0.3094; 55 kDa: F=0.3609, p=0.7817; 60 kDa: F=0.5898, p=0.6268; total: F=0.6937, p=0.5637). Significant outliers from each group were removed with the Grubb's test.

F. CCI injury did not significantly affect the pTau/tau ratio in the hippocampus; no effect of Posiphen (**Fig. 36**).

Fig. 36. **CCI injury did not significantly affect the pTau/tau ratio in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7, CCI/10 mg/kg Posiphen n=7-8). A Student's t-test between sham and vehicle groups revealed no significant differences in the ratio of pTau-S404/tau (50 kDa: t=0.2692, p=0.7910; 55 kDa: t=0.6487, p=0.5252; 60 kDa: t=0.3050, p=0.7641; total: t=0.2821, p=0.7813). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.064, p=0.3802; 55 kDa: F=0.5384, p=0.6599; 60 kDa: F=0.3292, p=0.8042; total: F=0.7124, p=0.5528). Significant outliers from each group were removed with the Grubb's test.

G. CCI injury did not significantly affect APP levels in the cerebral cortex; no effect of Posiphen (**Fig. 37**).

Fig. 37. **CCI injury did not significantly affect APP levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8-9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in APP (80 kDa: t=0.9267, p=0.3671; 160 kDa: t=0.0764, p=0.9400; total: t=1.082, p=0.2942). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=1.525, p=0.2281; 160 kDa: F=0.1097, p=0.9538; total: F=0.9482, p=0.4294). Significant outliers from each group were removed with the Grubb's test.

H. CCI injury significantly decreased aSyn levels in the cerebral cortex, an effect not reversed by Posiphen (**Fig. 38**).

Fig. 38. **CCI injury significantly decreased aSyn levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed a significant decrease in aSyn levels (t=3.267, *p=0.0045). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.9335, p=0.4362). Significant outliers from each group were removed with the Grubb's test.

I. CCI injury did not significantly affect BDNF in the cerebral cortex; no effect of Posiphen (**Fig. 39**).

Fig. 39. **CCI injury did not significantly affect BDNF in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9, CCI/vehicle n=6, CCI/2.5 mg/kg Posiphen n=6, CCI /5.0 mg/kg Posiphen n=4, CCI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=1.255, p=0.2314). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.429, p=0.2671). Significant outliers from each group were removed with the Grubb's test. Note that some animals were excluded due to a technical error.

J. CCI injury did not significantly affect Tau levels in the cortex; no effects of Posiphen (**Fig. 40**).

Fig. 40. **CCI injury did not significantly affect Tau levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=1.426, p=0.1721; 55 kDa: t=1.036, p=0.3147; 60 kDa: t=1.388, p=0.1830; total: t=1.401, p=0.1793). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.3204, p=0.8105; 55 kDa: F=1.452, p=0.2473; 60 kDa: F=0.6608, p=0.5824; total: F=1.071, p=0.3762). Significant outliers from each group were removed with the Grubb's test.

K. CCI injury did not significantly affect pTau-S404 levels in the cortex; no effects of Posiphen (**Fig. 41**).

Fig. 41. **CCI injury did not significantly affect pTau-S404 levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9-10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8-9, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=8-9). A Student's t-test between sham and vehicle groups revealed no significant differences in pTau-S404 levels (50 kDa: t=0.4931, p=0.6283; 55 kDa: t=0.1251, p=0.9020; 60 kDa: t=0.3674, p=0.7182; total: t=0.0236, p=0.9815). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.1179, p=0.9489; 55 kDa: F=0.5361, p=0.6613; 60 kDa: F=0.3874, p=0.7629; total: F=0.4195, p=0.7403). Significant outliers from each group were removed with the Grubb's test.

L. CCI injury did not significantly affect the pTau/tau ratio in the cortex; no effects of Posiphen (**Fig. 42**).

Fig. 42. **CCI injury did not significantly affect the pTau/tau ratio in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9-10, CCI/vehicle n=8, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=7-8). A Student's t-test between sham and vehicle groups revealed no significant differences in the pTau-S404/tau ratio (50 kDa: t=1.406, p=0.1790; 55 kDa: t=1.496, p=0.1553; 60 kDa: t=1.359, p=0.1942; total: t=1.482, p=0.1589). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.1231, p=0.3068; 55 kDa: F=1.318, p=0.2891; 60 kDa: F=1.157, p=0.3436; total: F=1.331, p=0.2840). Significant outliers from each group were removed with the Grubb's test.

Discussion:

Contrary to LFP, CCI did not increase APP in the hippocampus. However, two effects were observed after CCI that were not observed after LFP: a decrease in tau 55kDa in hippocampus and alpha-synuclein in cerebral cortex. Those effects were unexpected and may correspond to a dysfunction of neurons directly impacted by the injury. As we observed with biochemical changes induced by LFP, Posiphen had no effect. Unfortunately, to conserve animals, no histology was included in the SOW for the CCI groups. Accordingly, we could not confirm in this group the effects of Posiphen observed after LFP on dopaminergic terminals.

4. KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that Posiphen at 3 concentrations is well tolerated in rats and did not induce unwanted cognitive, histological or biochemical effects even when administered immediately after two different kinds of TBI, i.e. during the period corresponding of heightened sensitivity to a secondary insult following injury.
- Demonstration that Posiphen protects against TBI-induced loss of tyrosine hydroxylase in striatum, an index of damage to the terminals of nigrostriatal dopaminergic neurons that are the main cells affected in Parkinson's disease.
- Demonstration that high doses of Posiphen have a mild anti-inflammatory effect against TBI-induced microglial activation.

5. CONCLUSION

Our results provide preclinical evidence that Posiphen has beneficial effects on neuronal damage induced by TBI. The main finding concerned the nigrostriatal dopaminergic system, which is affected in Parkinson's disease, a severe neurodegenerative disorder for which TBI may be a risk factor. This supports further development of Posiphen for the prevention of long-term detrimental effects of TBI on brain neurons.

We did not observe any improvement of TBI-induced APP increases in the hippocampus as expected. However, this result is in agreement with more recent results from QR Pharma, further suggesting that the main effect of Posiphen in brain is not related to its ability to decrease APP.

While our results confirmed that even mild TBI produces alterations in brain, we did not observe cognitive deficits after either type of TBI, and accordingly were unable to determine whether Posiphen produced functional benefits. We suspect that experimental conditions involving daily handling and injections may have interfered with our ability to measure such deficits post injury.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

Nothing to report to date

7. INVENTIONS, PATENTS, AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

Upon completion of Tasks 1 and 2, we have determined that a month long treatment with Posiphen at doses of 2.5, 5, and 10 mg/kg (i.p.) is well tolerated in rats. Furthermore, we showed a reversal of a significant decrease in TH+ terminals in the striatum (all doses) and an attenuation of injury-induced microglial activation (10 mg/kg dose) in animals subjected to TBI induced by LFP.

9. OTHER ACHIEVEMENTS

Three post-doctoral fellows who have completed their post-doctoral training under this award have obtained new positions:

Garima Dutta, PhD: scientific Liaison by Banyan Biomarkers, a Biotech company in San Diego CA.
Sudhakar Subramaniam, PhD: Assistant Researcher, University of California, Irvine.
Aroa Relano-Gines, PhD: Project Scientist, Chesselet lab at UCLA.

Two post-doctoral Fellows have continued their post-doctoral training under this award:

Asa Hatami, PhD: Post-doctoral Fellow with Dr. Peter Butler at UCLA.
Kimberly McDowell, PhD continues training in the Chesselet lab at UCLA.

A neurosurgeon from Japan, Dr. Nobuo Kutsuna obtained research training under this award. Dr. Kutsuna returned to Japan to complete his neurosurgical training in Tokyo.

Several UCLA undergraduate students trained in research by participating in studies supported by this award:

Sonoko Kawakatsu, BS, now a student in the School of Pharmacy, University of California San Diego
Benjamin Boodaie, BS, now a medical student in the Ichan School of Medicine, Mount Sinai, New York
Anna Kosmalska, BS, is applying to Schools of Physical Therapy
Akash Patel is finishing his degree at UCLA and applying to medical school

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11. APPENDICES

Appendix 1 – Figures and tables with legends

APPENDIX 1: Figures

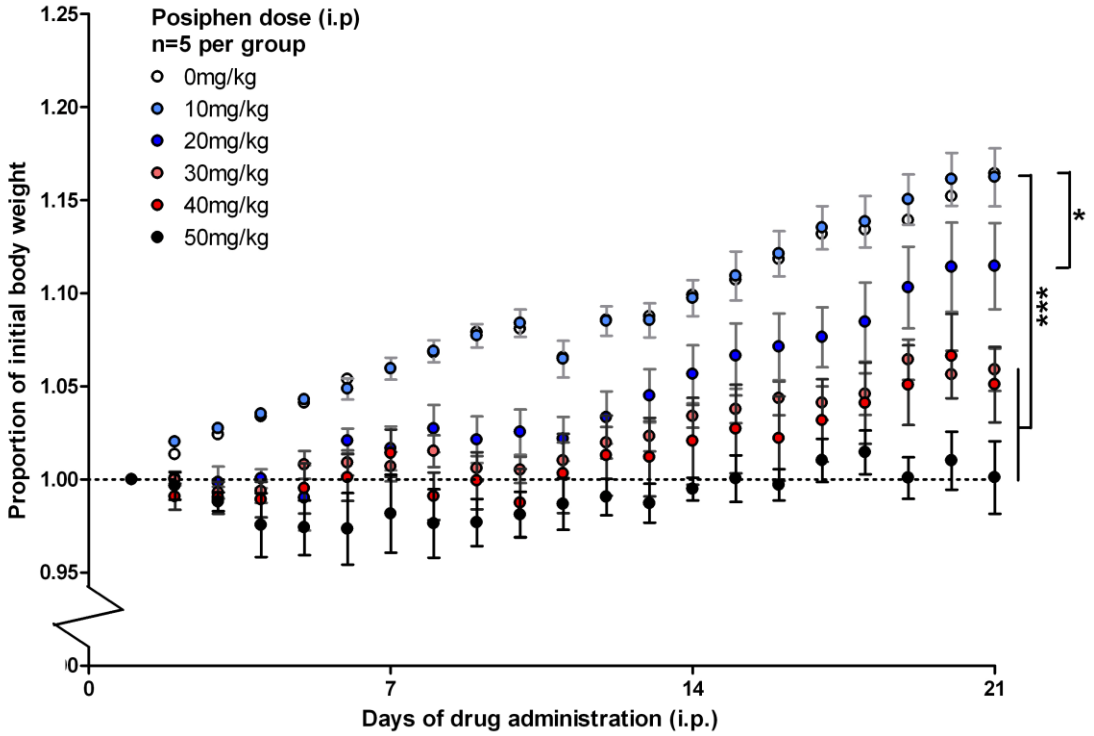


Fig. 1: **Body weight of rats administered vehicle or Posiphen i.p. for 21 days.** Mean+/-SEM (n=5 per group) proportion of body weight on each day of treatment compared to day 1 of treatment. Repeated measures two-way ANOVA: treatment effect F=13.42, p<0.001; day effect F=106.091, p<0.001; interaction of treatment and day F=5.676, p<0.001. Fisher's LSD post-hoc test for groups shown compared to vehicle or Posiphen 10 mg/kg: *p<0.05, ***p<0.001 for days 8-21.

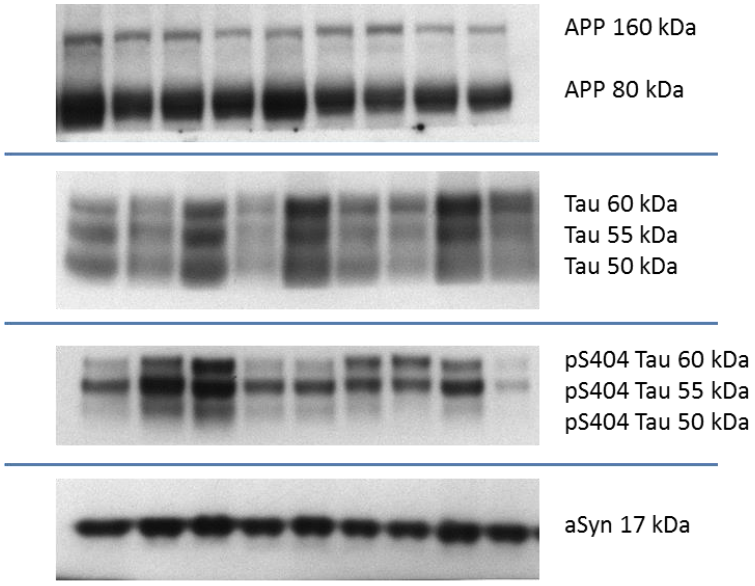


Fig. 2. **Representative images of immunoblotting for APP, tau, p-tau, and aSyn in brain tissue**. The following antibodies were used: anti-APP at 1:1,000 (clone 22C11, Millipore, #MAB348); anti-aSyn at 1:3,000 (BD Biosciences, #610787), anti-tau at 1:10,000 (Abcam, Ab76128); and anti-phospho-S404-tau at 1:3,000 (Abcam, Ab92676).

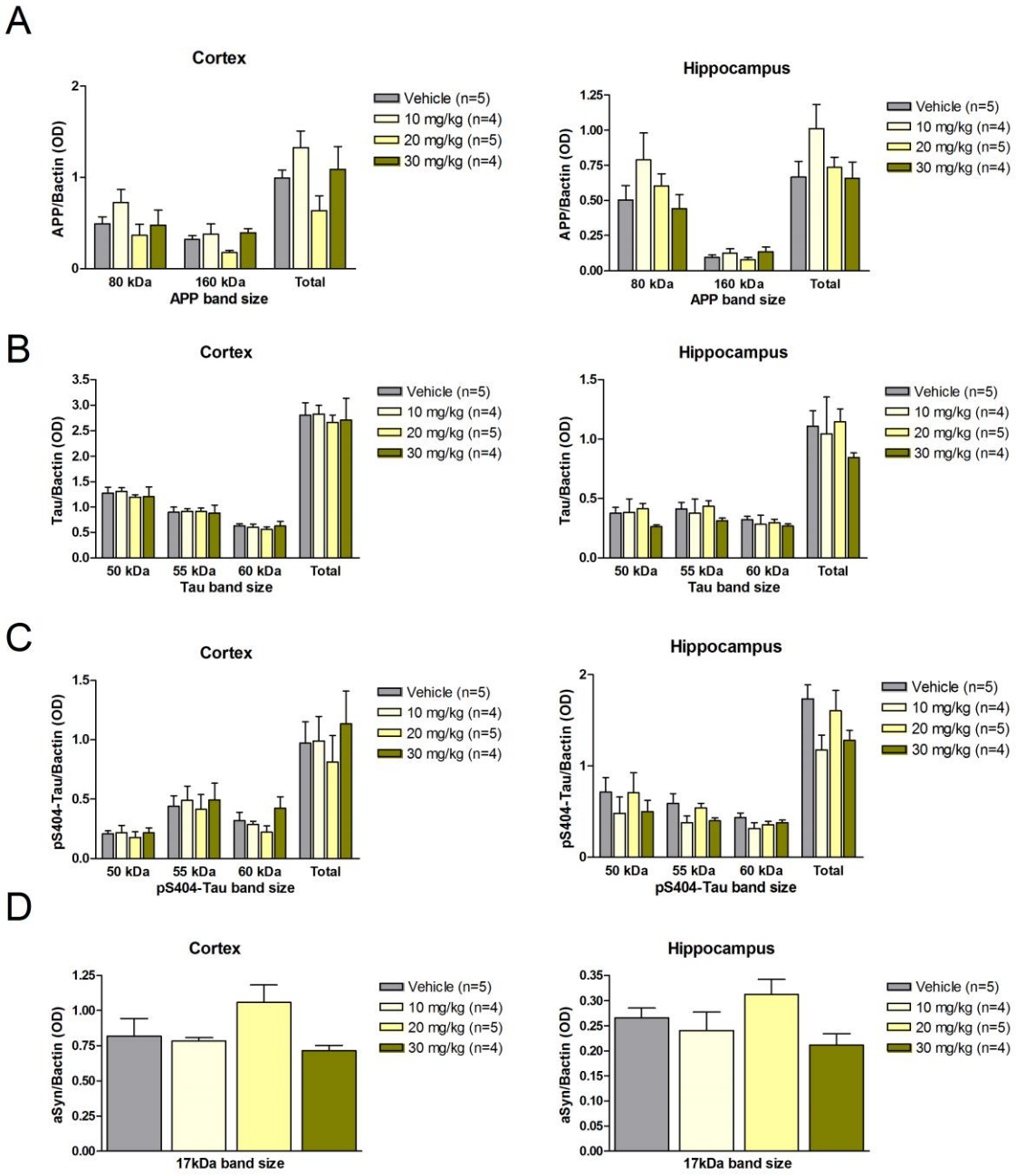


Fig. 3. **Posiphen did not alter the levels of APP, tau, p-tau, and aSyn in the hippocampus or cortex of uninjured rats.** Data are shown as the mean +/- S.E.M (vehicle n=5, 10 mg/kg n=4, 20 mg/kg n=5, 30 mg/kg n=4). Significant outliers from each group were removed with the Grubb's test. **(A)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in APP levels between the vehicle and Posiphen-treated groups (Cortex: 80 kDa: F=1.387, p=0.2877; 160 kDa: F=2.920, p=0.0710; total: F=2.841, p=0.0759; Hippocampus: 80 kDa: F=1.448, p=0.2711; 160 kDa: F=1.413, p=0.2805; total: F=1.850, p=0.1845). **(B)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in tau levels between the vehicle and Posiphen-treated groups (Cortex: 50 kDa: F=0.2584, p=0.8541; 55 kDa: F=0.0282, p=0.9933; 60 kDa: F=0.3276, p=0.8054; total: F=0.0877, p=0.9656; Hippocampus: 50 kDa: F=0.9768, p=0.4315; 55 kDa: F=0.6443, p=0.5993; 60 kDa: F=0.3239, p=0.8080; total: F=0.6190, p=0.6142). **(C)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in pS404-Tau levels between the vehicle and Posiphen-treated groups (Cortex: 50 kDa: F=0.1761, p=0.9108; 55 kDa: F=0.1065, p=0.9549; 60 kDa: F=1.608, p=0.2322; total: F=0.3552, p=0.7862; Hippocampus: 50 kDa: F=0.4938, p=0.6924; 55 kDa: F=1.748, p=0.2032; 60 kDa: F=1.033, p=0.4083; total: F=2.279, p=0.1242). **(D)** A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences in aSyn levels between the vehicle and Posiphen-treated groups (Cortex: F=2.210, p=0.1322; Hippocampus: F=2.410, p=0.1105).

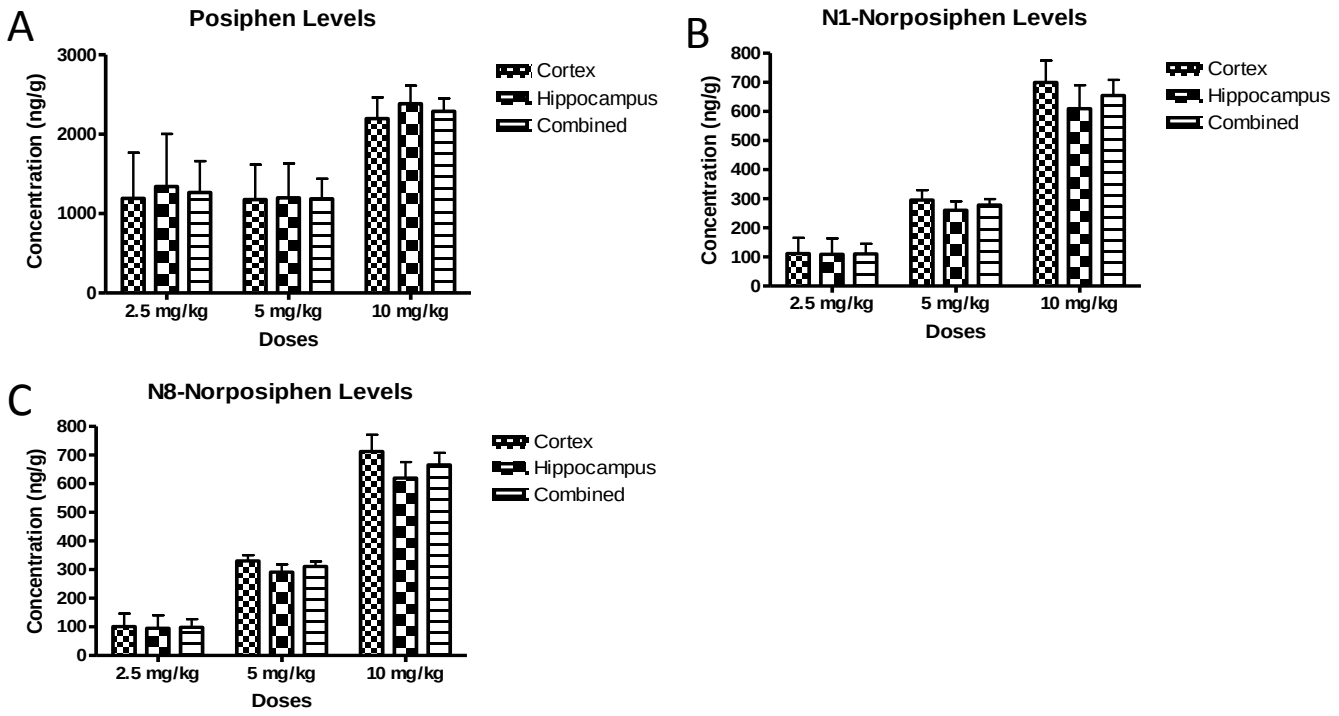


Fig. 4. **Levels of Posiphen, N1-Norposiphen and N8-Norposiphen were measured in hippocampal and cortical brain tissue (n=3 per group).** (A) Posiphen levels, cortical samples p=0.157, hippocampal samples p=0.143, combined samples p= 0.009; (B) N1-Norposiphen levels, cortical samples p=0.004, hippocampal samples p=0.007, combined samples p= 0.001; (C) N8-Norposiphen levels, cortical samples p=0.003, hippocampal samples p=0.004, combined samples p= 0.001; ANOVA.

		Vehicle	2.5 mg/kg	5 mg/kg	10 mg/kg	PBS
# of Total Assigned Animals	FPI	26	24	23	30	
	SHAM					22
# Deaths	FPI	5	2	1	6	
	SHAM					1
# Suboptimal Surgery/Injury	FPI	1	2	1	4	
	SHAM					1
# Excluded (SA performance)	FPI	4	4	6	6	
	SHAM					5
FINAL GROUP #	FPI	16	16	15	14	
	SHAM					15

Table 1. **FPI group assignment.** A total of 125 rats were originally assigned to the study. During the course of the study period, a total of 15 rats died. After review of the surgical conditions, 9 animals were excluded from subsequent analysis (i.e., suboptimal injury with a short loss of consciousness). Lastly, 25 animals were excluded from subsequent analysis based on their poor/lack of performance on the spatial alternations (SA) behavioral test. These exclusion criteria consisted of a pre-treatment SA score of less than 57% or less than 11 total arm entries (e.g., did not complete the test). Thus, the final groups for analyses were: 16 rats in the FPI/Vehicle group, 16 rats in the FPI/2.5 mg/kg Posiphen group, 15 rats in the FPI/5.0 mg/kg Posiphen group, 14 rats in the FPI/10 mg/kg Posiphen group, and 15 rats in the Sham FPI/Vehicle group.

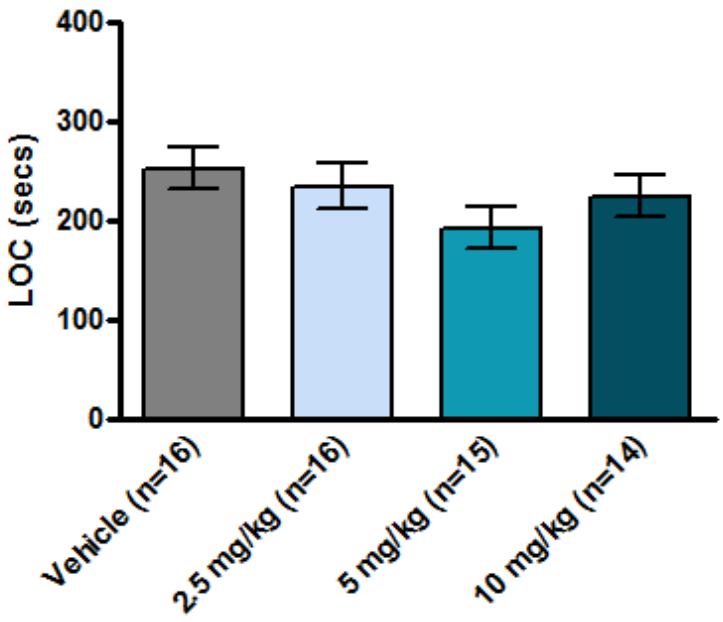


Fig. 5. **Mean loss of consciousness (LOC) for the FPI groups.** Data are shown as the mean +/- S.E.M (FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=16, FPI/5.0 mg/kg Posiphen n=15, FPI/10 mg/kg Posiphen n=14). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between groups (F=1.375, p=0.2597).

Fig. 6. **Image of the maze used for the spatial alternation task.** Rats were placed in the middle of the maze and left to explore for 15 minutes. The number and sequence of arm entries were recorded for calculation of a percent alternation score. An alternation consists of 4 different arm choices of 5 consecutive arm entries. A 4/5 percent alternation score was computed by dividing the number of observed alternations in overlapping quintuplets by the number of possible alternations and multiplying the quotient by 100.

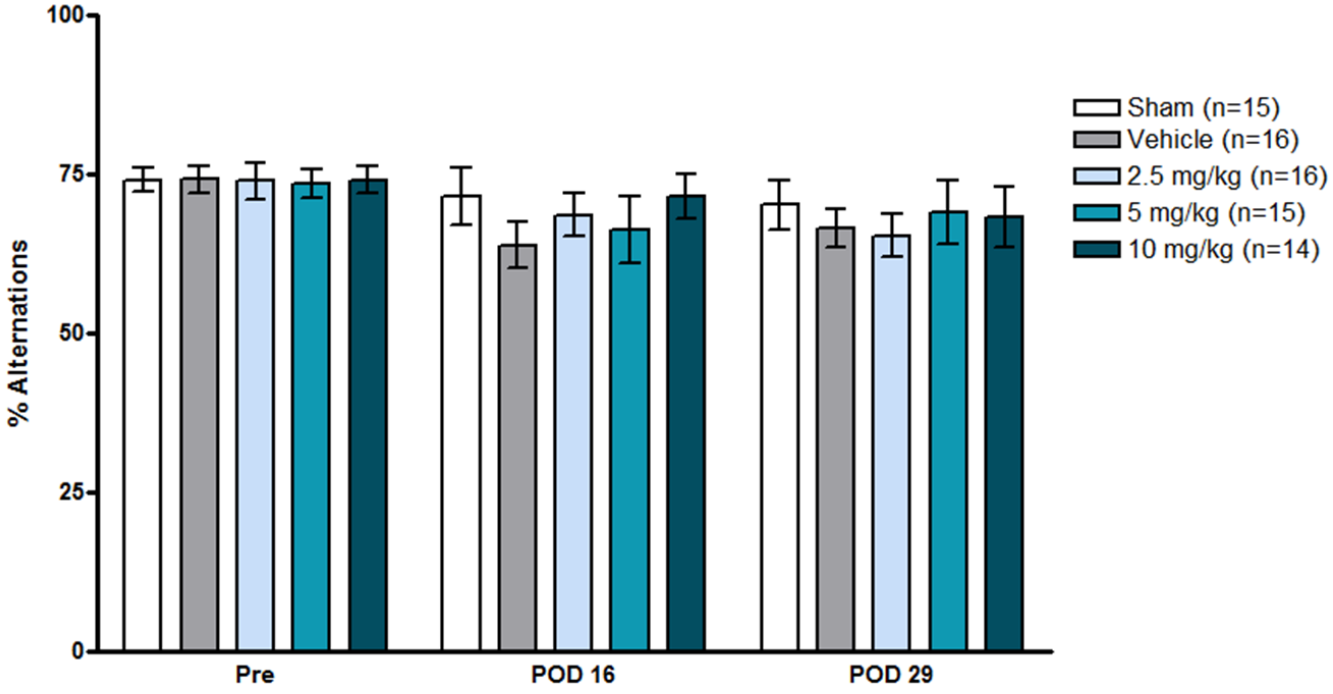


Fig. 7. **No effect of LFP injury on spatial alternations.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=16, FPI/5.0 mg/kg Posiphen n=15, FPI/10 mg/kg Posiphen n=14). A two-way repeated-measures ANOVA with Bonferroni post-hoc comparisons revealed a significant effect of time ($F=5.319$, $p=0.0062$), but no significant differences between the vehicle and Posiphen-treated groups ($F=0.2866$, $p=0.8349$). Planned comparisons (sham vs. vehicle) within each time point also revealed no significant differences. POD = post-operative day.

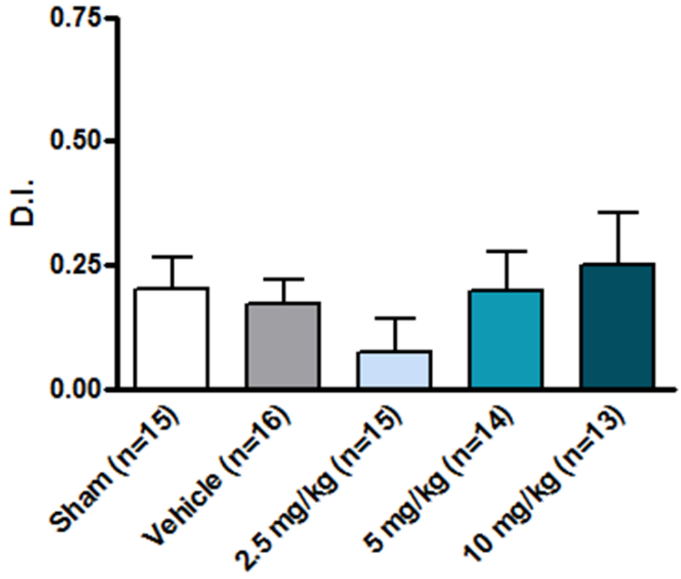


Fig. 8. **No effect of LFP injury on novel object recognition** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=15, FPI/5.0 mg/kg Posiphen n=14, FPI/10 mg/kg Posiphen n=13). A Student's t-test between sham and vehicle groups revealed no significant differences ($t=0.3645$, $p=0.7181$). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups ($F=0.9716$, $p=0.4130$). Note that data from 1 animal each from the 2.5, 5, and 10 mg/kg groups were not used because both objects were not explored during testing. DI = discrimination index.

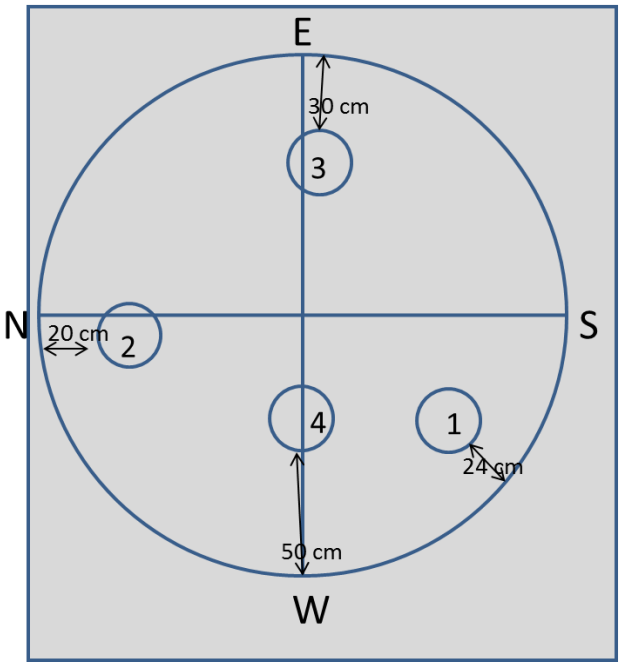


Fig. 9. **Diagram of the platform positions and release points used in the Morris water maze.** For a total of 3 days, rats were tested on their ability to find 4 platform positions, using paired trials (8 total trials per day). The platform positions (1, 2 3 and 4) were switched for every trial. N, S, E and W were the release points (Fig. Rats were allowed a maximum swim time of 90 seconds (guided to platform if needed after 90 sec). Trial 2 (T2) was started 5 seconds later. The time between Trial 1 (T1) at each new platform position was 5 min. The percent improvement in finding the platform was calculated using the following formula: 1 – T2/T1 * 100%.

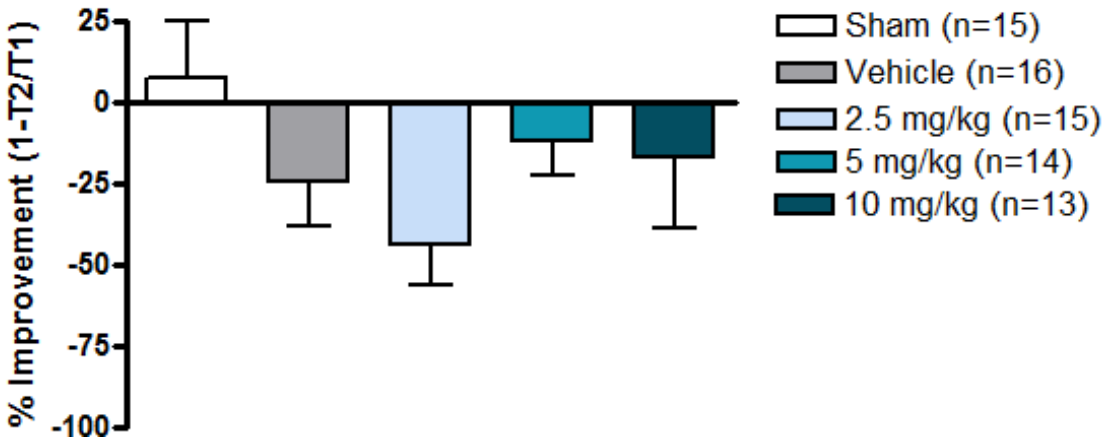


Fig. 10. **No effect of LFP injury on working memory in the Morris water maze.** Averaged data from the 3 testing days are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=15, FPI/vehicle n=16, FPI/2.5 mg/kg Posiphen n=15, FPI/5.0 mg/kg Posiphen n=14, FPI/10 mg/kg Posiphen n=13). A Student's t-test between sham and vehicle groups revealed no significant differences (t=1.445, p=0.1592). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.8984, p=0.4481). Note that data from 1 animal each from the 2.5, 5, and 10 mg/kg groups were not used because of a technical error during testing.

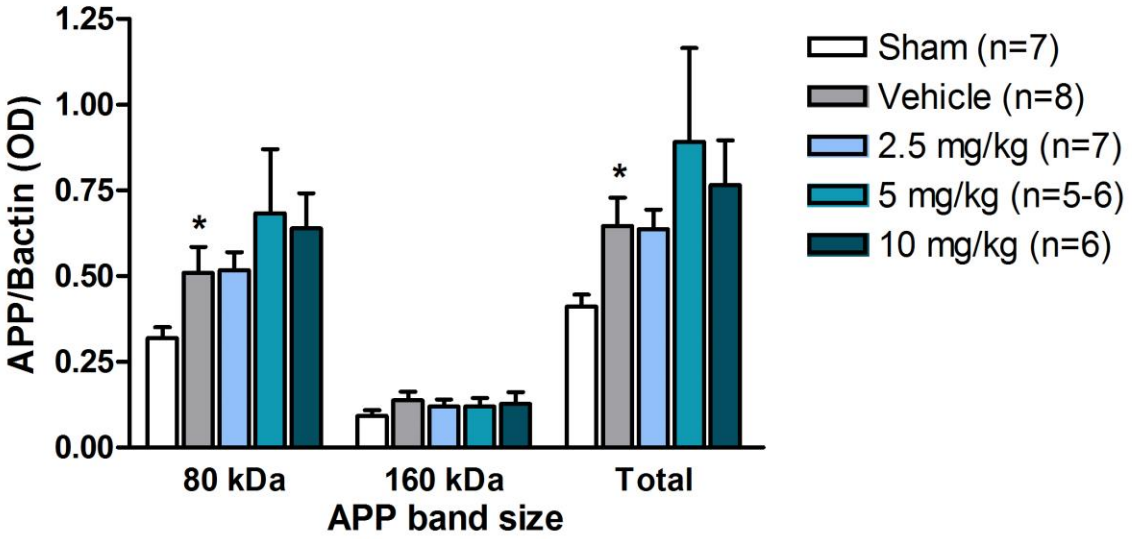


Fig. 11. **LFP injury significantly increased APP levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed a significant increase in the 80 kDa band of APP and total APP levels in injured rats (80 kDa: t=2.222, *p=0.0446; 160 kDa: t=1.444, p=0.1723; total: t=2.533, *p=0.0250). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.6501, p=0.5909; 160 kDa: F=0.1132, p=0.9515; total: F=0.6569, p=0.5869). Significant outliers from each group were removed with the Grubb's test.

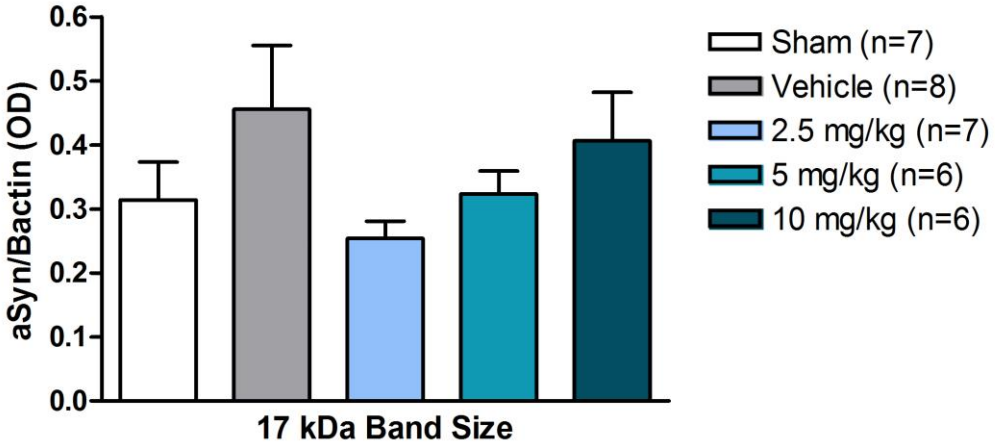


Fig. 12. **LFP injury did not significantly affect aSyn levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=1.173, p=0.2618). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.658, p=0.2038). Significant outliers from each group were removed with the Grubb's test.

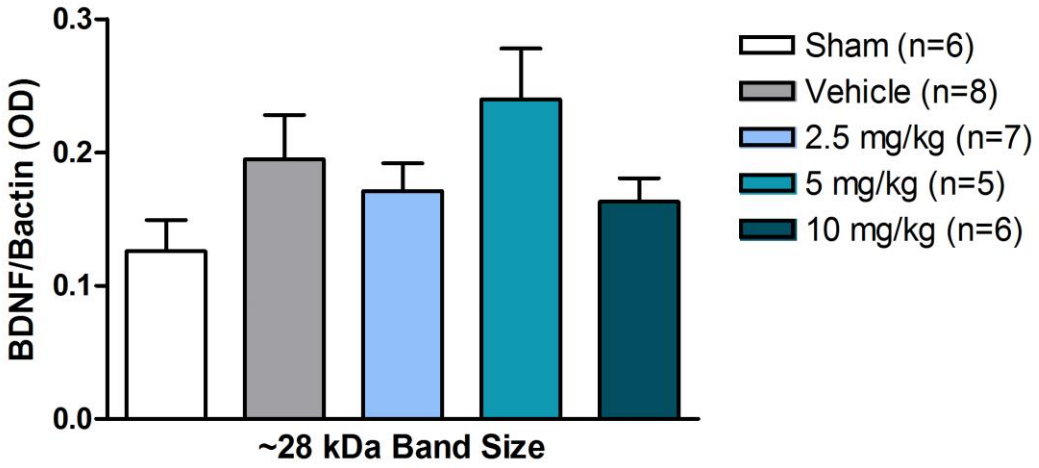


Fig. 13. **LFP injury did not significantly affect BDNF levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=1.579, p=0.1402). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.205, p=0.3313). Significant outliers from each group were removed with the Grubb's test.

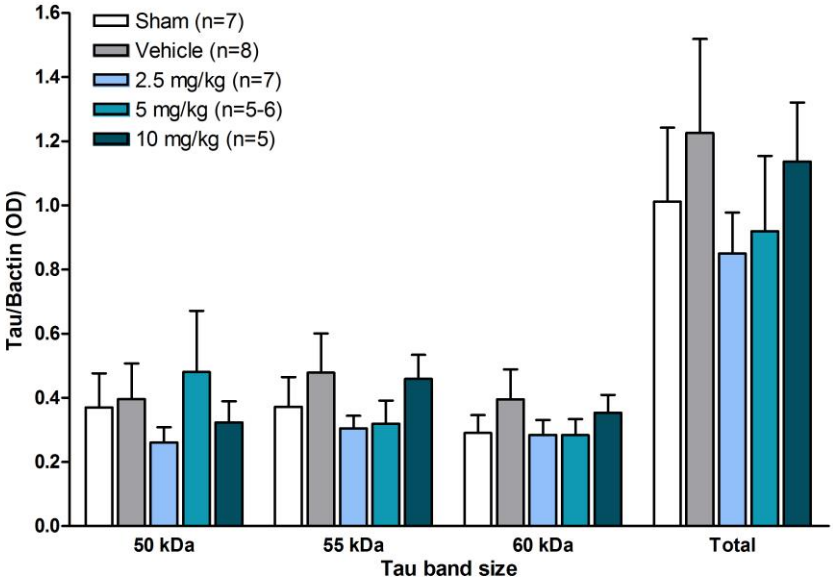


Fig. 14. **LFP injury did not significantly affect Tau levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=0.1708, p=0.8670; 55 kDa: t=0.6774, p=0.5100; 60 kDa: t=0.9264, p=0.3711; total: t=0.5609, p=0.5844). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.6467, p=0.5933; 55 kDa: F=1.017, p=0.4050; 60 kDa: F=0.6257, p=0.6064; total: F=0.6232, p=0.6079). Significant outliers from each group were removed with the Grubb's test.

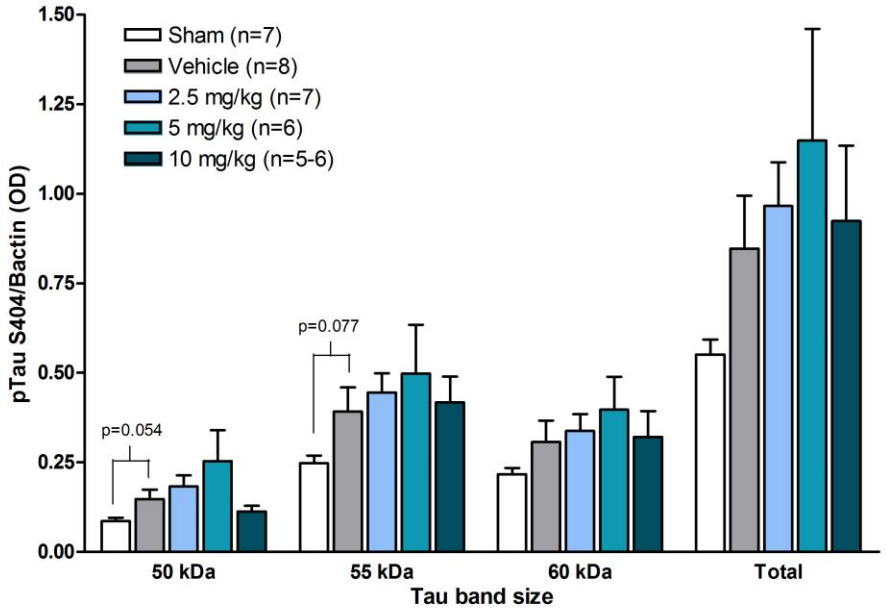


Fig. 15. **LFP injury showed a strong trend towards increased pTau-S404 levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=5-6). A Student's t-test between sham and vehicle groups revealed a strong trend toward a significant increase in the pS404-tau 50 and 55 kDa bands in injured rats (50 kDa: t=2.116, p=0.0542; 55 kDa: t=1.922, p=0.0768; 60 kDa: t=1.350, p=0.2000; total: t=1.810, p=0.0935). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.489, p=0.2450; 55 kDa: F=0.2879, p=0.8336; 60 kDa: F=0.3299, p=0.8037; total: F=0.4063, p=0.7499). Significant outliers from each group were removed with the Grubb's test.

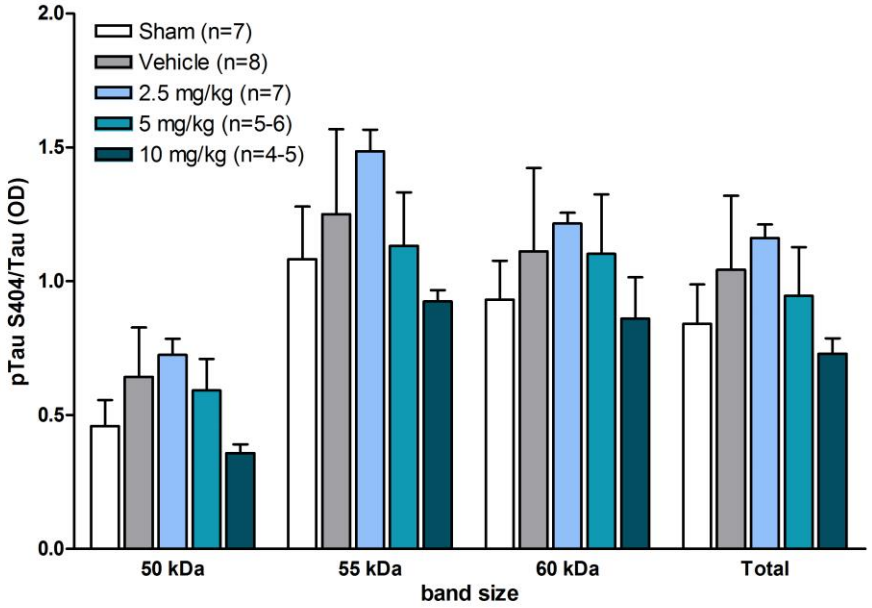


Fig. 16. **LFP injury did not significantly affect the pTau/tau ratio in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=5-6, FPI/10 mg/kg Posiphen n=4-5). A Student's t-test between sham and vehicle groups revealed no significant differences in the ratio of pTau-S404/tau (50 kDa: t=0.8417, p=0.4152; 55 kDa: t=0.4350, p=0.6707; 60 kDa: t=0.4995, p=0.6258; total: t=0.6208, p=0.5455). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.011, p=0.4076; 55 kDa: F=1.007, p=0.4092; 60 kDa: F=0.3787, p=0.7694; total: F=0.7955, p=0.5101). Significant outliers from each group were removed with the Grubb's test.

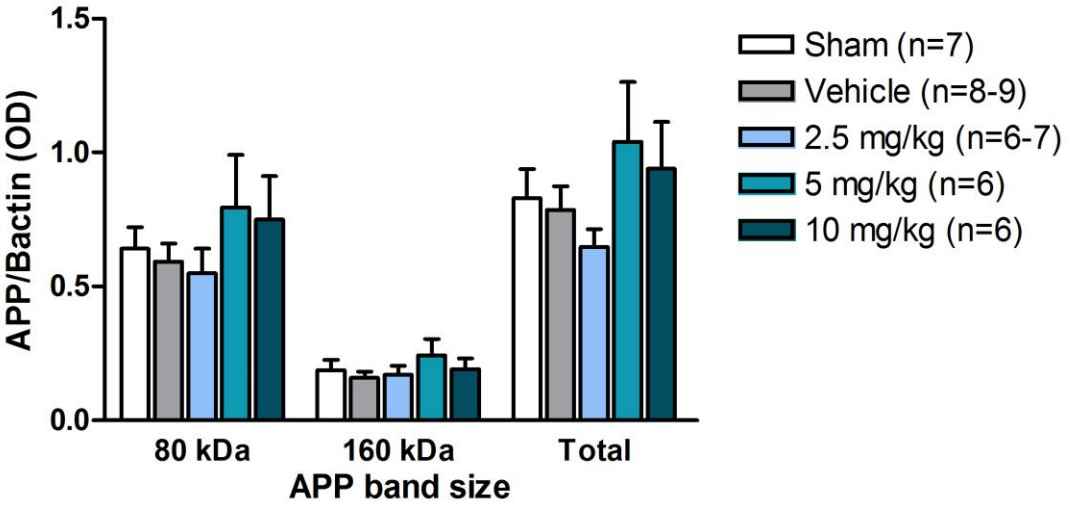


Fig. 17. **LFP injury did not significantly affect APP levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8-9, FPI/2.5 mg/kg Posiphen n=6-7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences (80 kDa: t=0.4624, p=0.6509; 160 kDa: t=0.5134, p=0.6720; total: t=0.3077, p=0.7628). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.8791, p=0.4658; 160 kDa: F=0.8770, p=0.4681; total: F=1.346, p=0.2841). Significant outliers from each group were removed with the Grubb's test.

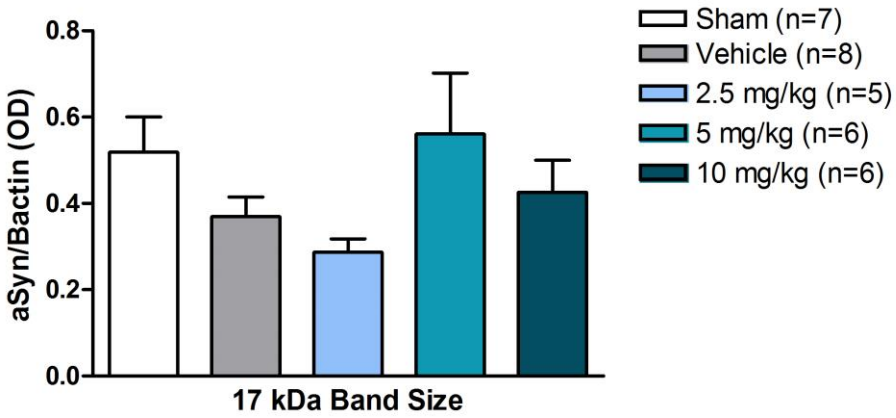


Fig. 18. **LFP injury did not significantly affect aSyn levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=5, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=1.662, p=0.1204). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.940, p=0.1526). Significant outliers from each group were removed with the Grubb's test.

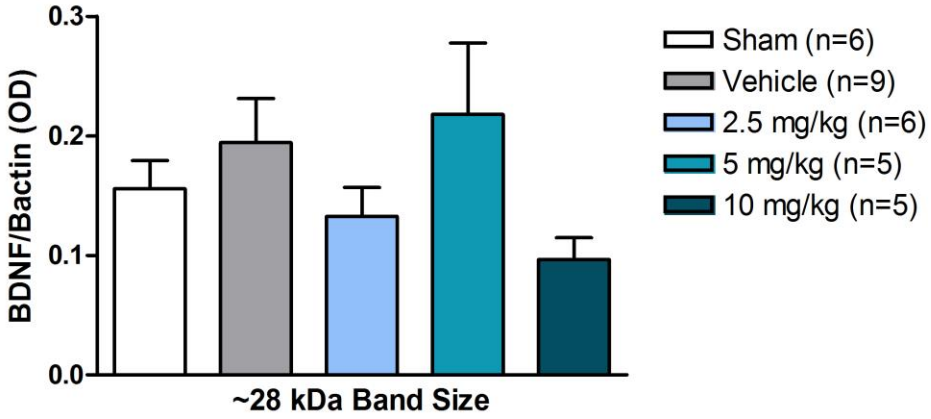


Fig. 19. **LFP injury did not significantly affect BDNF in the cortex.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6, FPI/vehicle n=9, FPI/2.5 mg/kg Posiphen n=6, FPI/5.0 mg/kg Posiphen n=5, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=0.7770, p=0.4511). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.923, p=0.1553). Significant outliers from each group were removed with the Grubb's test.

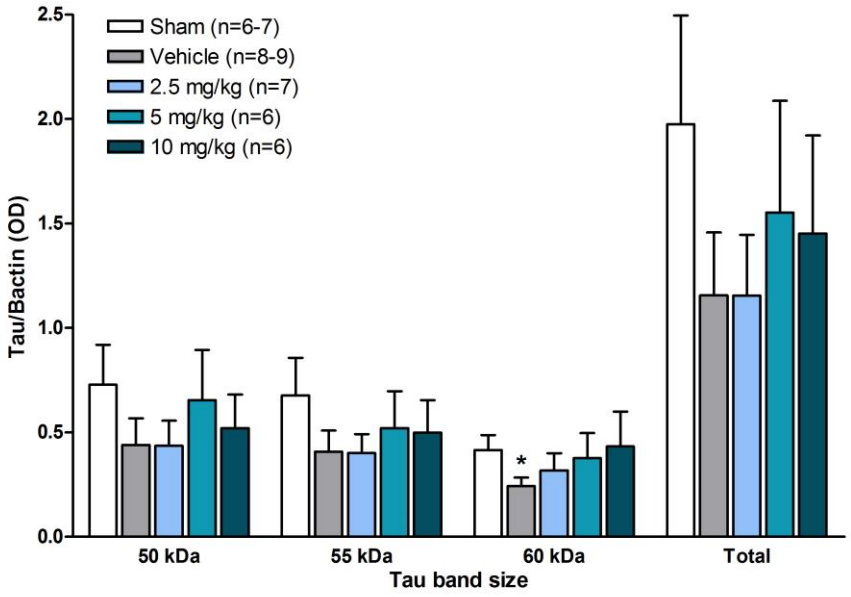


Fig. 20. **LFP injury significantly decreased Tau levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=6-7, FPI/vehicle n=8-9, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups a significant decrease in the 60 kDa tau band in injured rats (50 kDa: t=1.297, p=0.2157; 55 kDa: t=1.387, p=0.1872; 60 kDa: t=2.259, *p=0.0433; total: t=1.440, p=0.1719). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.3838, p=0.7656; 55 kDa: F=0.2250, p=0.8781; 60 kDa: F=0.6425, p=0.5955; total: F=0.2697, p=0.8466). Significant outliers from each group were removed with the Grubb's test.

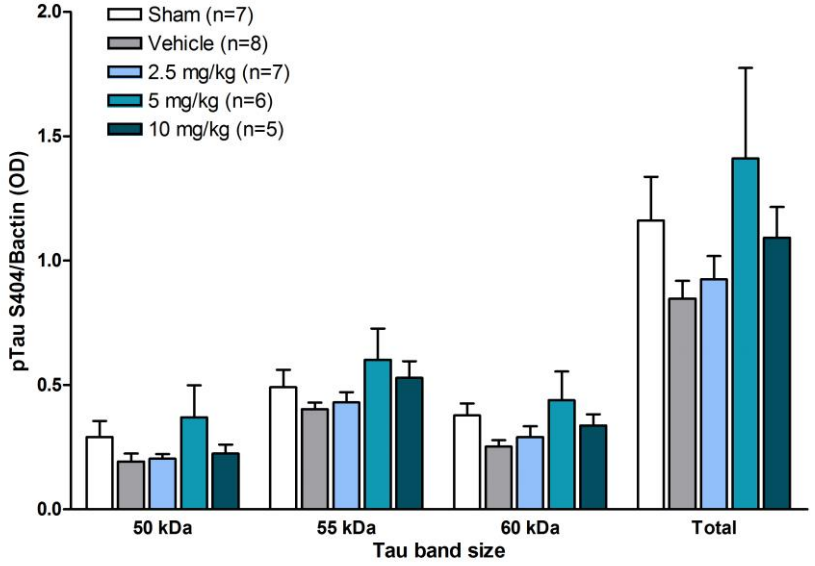


Fig. 21. **LFP injury did not significantly affect pTau-S404 levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=5). A Student's t-test between sham and vehicle groups revealed no significant differences in pTau-S404 levels (50 kDa: t=0.1943, p=0.8487; 55 kDa: t=0.0619, p=0.9515; 60 kDa: t=0.6874, p=0.5031; total: t=0.2330, p=0.8191). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.7531, p=0.5318; 55 kDa: F=0.5570, p=0.6487; 60 kDa: F=0.7203, p=0.5501; total: F=0.6714, p=0.5783). Significant outliers from each group were removed with the Grubb's test.

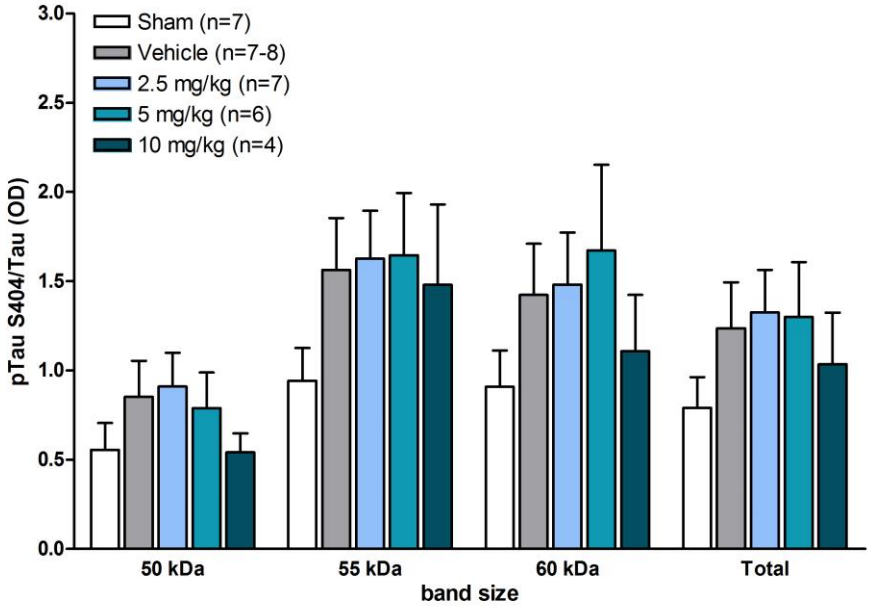


Fig. 22. **LFP injury did not significantly affect the pTau/tau ratio in the cortex.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=7, FPI/vehicle n=7-8, FPI/2.5 mg/kg Posiphen n=7, FPI/5.0 mg/kg Posiphen n=6, FPI/10 mg/kg Posiphen n=4). A Student's t-test between sham and vehicle groups revealed no significant differences in the pTau-S404/tau ratio (50 kDa: t=1.151, p=0.2706; 55 kDa: t=1.743, p=0.1049; 60 kDa: t=1.457, p=0.1708; total: t=1.391, p=0.1877). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.5130, p=0.6777; 55 kDa: F=0.0411, p=0.9886; 60 kDa: F=0.3415, p=0.7955; total: F=0.1697, p=0.9156). Significant outliers from each group were removed with the Grubb's test.

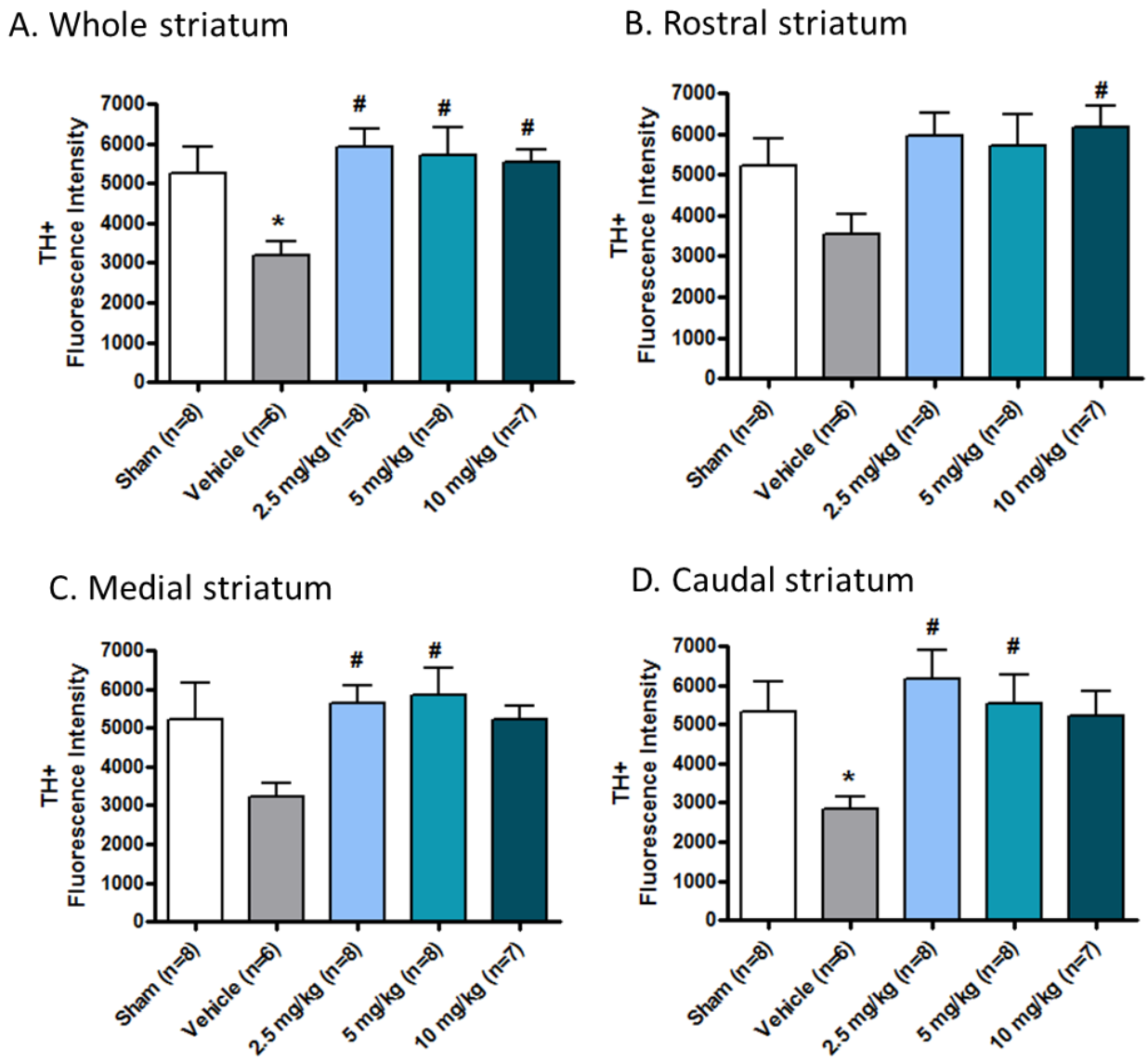


Fig. 23. **Treatment with Posiphen significantly attenuated the FPI-induced decrease in TH+ terminals in the ipsilateral striatum.** Data are shown as the mean +/- S.E.M. (Sham FPI/vehicle n=8, FPI/vehicle n=6, FPI/2.5 mg/kg Posiphen n=8, FPI/5.0 mg/kg Posiphen n=8, FPI/10 mg/kg Posiphen n=7). **(A)** A Student's t-test between sham and vehicle groups revealed a significant decrease in TH-ir in the striatum (t=2.470, *p=0.0295). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in all Posiphen-treated groups when compared with the vehicle (F=5.499, #p<0.05 when compared with vehicle). **(B)** A Student's t-test between sham and vehicle groups revealed a strong trend toward a decrease in TH-ir in the rostral striatum (t=1.960, p=0.0736). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 10 mg/kg Posiphen when compared with the vehicle (F=3.310, #p<0.05 when compared with vehicle). **(C)** A Student's t-test between sham and vehicle groups revealed no significant differences in TH-ir in the medial striatum (t=1.756, p=0.1045). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 2.5 and 5 mg/kg Posiphen when compared with the vehicle (F=4.605, #p<0.05 when compared with vehicle). **(D)** A Student's t-test between sham and vehicle groups revealed a significant decrease in TH-ir in the caudal striatum (t=2.667, *p=0.0205). A one-way ANOVA with Bonferroni post-hoc comparisons revealed a significant increase in TH-ir in rats treated with 2.5 and 5 mg/kg Posiphen when compared with the vehicle (F=4.414, #p<0.05 when compared with vehicle). Significant outliers from each group were removed with the Grubb's test.

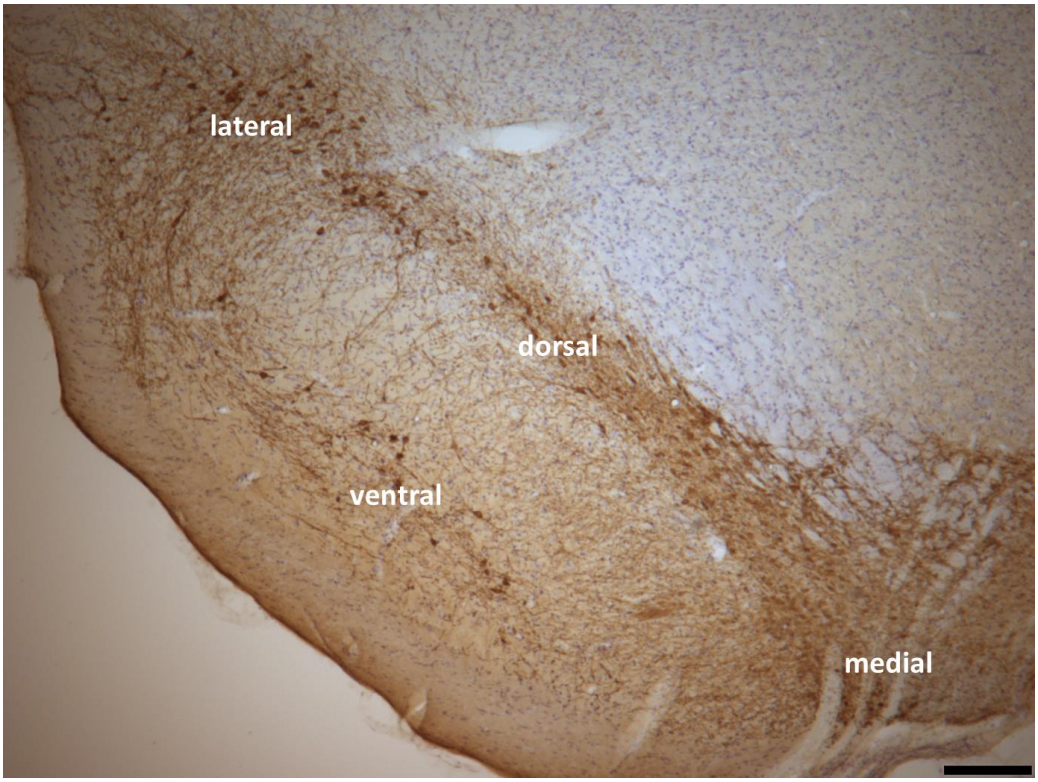


Fig. 24. **Representative image of brain tissue processed for TH-immunoreactivity with a Nissl counterstain**. Subregions of the substantia nigra are marked with white text. The image was taken at 5X (Scale bar = 200 um).

Brain Region	Endpoint Measure	Injury effect (Sham vs. Vehicle)	Treatment effect (Vehicle vs. Posiphen)	
		Student's t-test Overall P value	One-way ANOVA Overall P value	Bonferroni Post-hoc tests
Whole SN	TH+ neurons	0.1985	0.0971	not significant
	TH+ and Nissl+ neurons	0.9716	0.1239	not significant
Dorsal SN	TH+ neurons	0.2582	0.2113	not significant
	TH+ and Nissl+ neurons	0.5893	0.0500	not significant
Lateral SN	TH+ neurons	0.4369	0.4464	not significant
	TH+ and Nissl+ neurons	0.9950	0.4530	not significant
Medial SN	TH+ neurons	0.7066	0.6517	not significant
	TH+ and Nissl+ neurons	0.4861	0.3118	not significant
Ventral SN	TH+ neurons	0.1181	0.2655	not significant
	TH+ and Nissl+ neurons	0.5621	0.7272	not significant

Table 2. **Stereological cell estimates of the substantia nigra.** LFP injury does not significantly affect the number of TH+ or Nissl+ neurons in the ipsilateral substantia nigra. Groups include: Sham FPI/Vehicle (n=8), FPI/Vehicle (n=7), FPI/2.5 mg/kg Posiphen (n=9), FPI/5.0 mg/kg Posiphen (n=9), FPI/10 mg/kg Posiphen (n=6-7). Significant outliers from each group were removed with the Grubb's test.

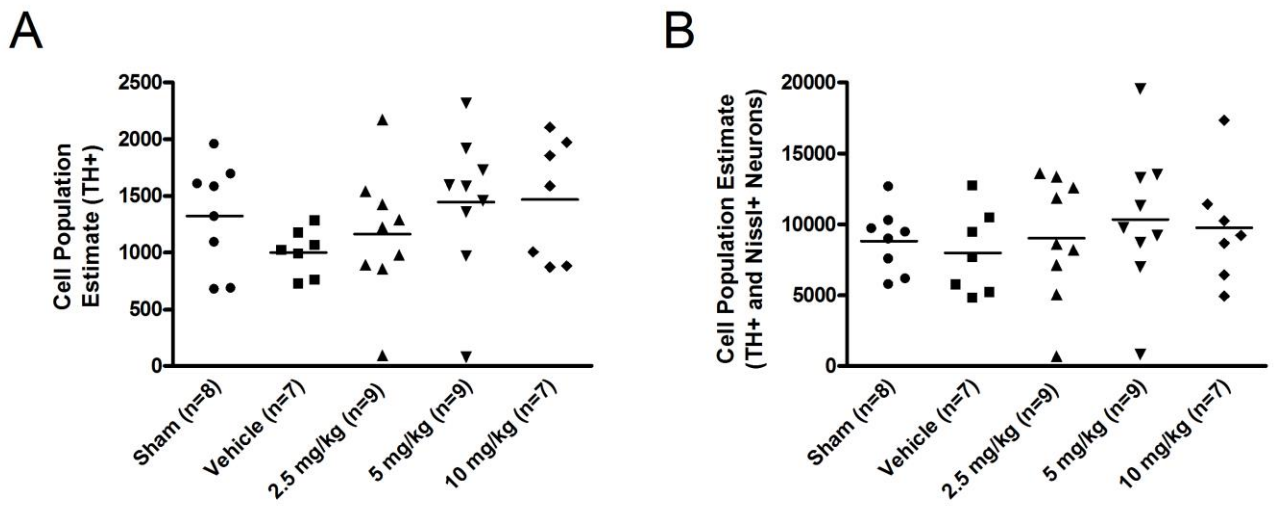
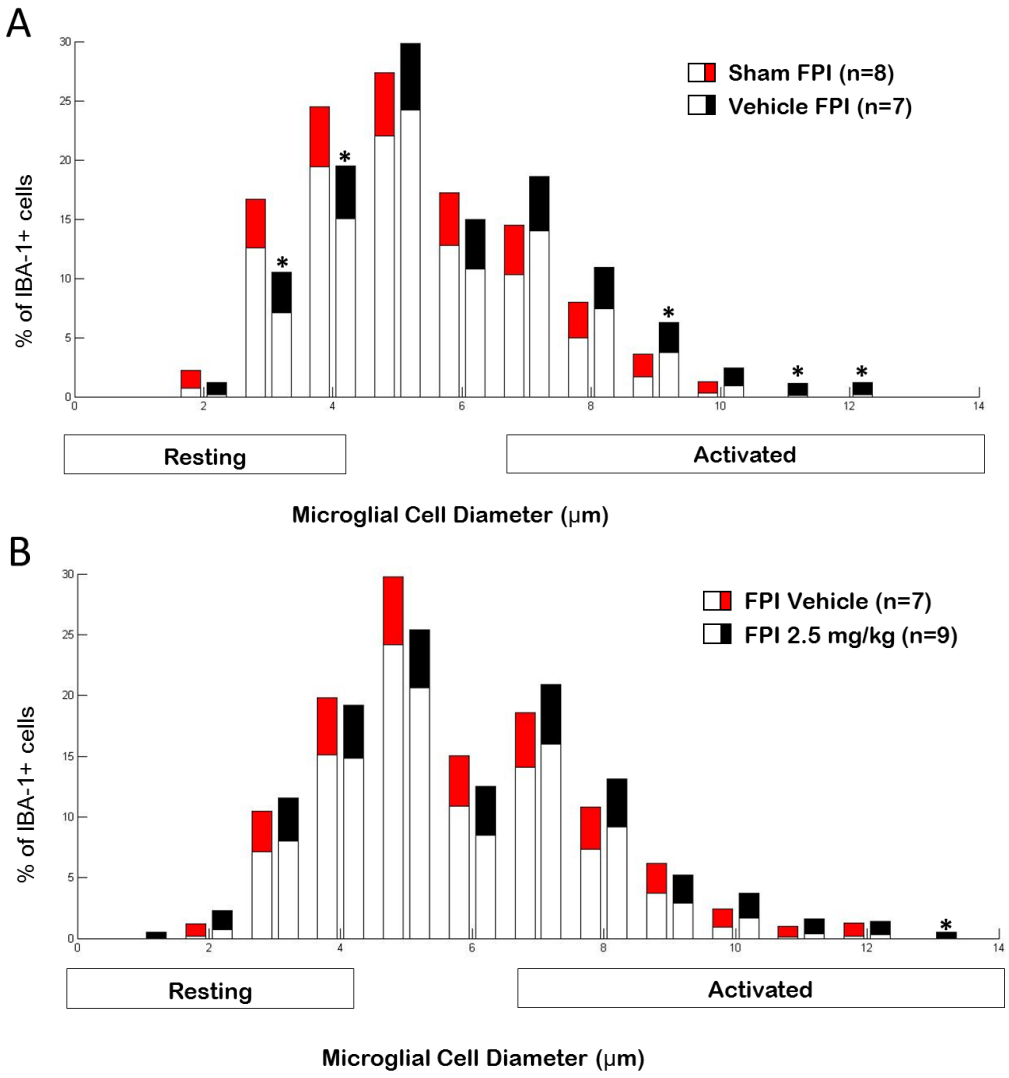


Fig. 25. Scatter plot of the cell estimates for TH+ neurons (A) and the TH+/Nissl+ neurons (B) in the ventral SN. Groups include: Sham FPI/Vehicle (n=8), FPI/Vehicle (n=7), FPI/2.5 mg/kg Posiphen (n=9), FPI/5.0 mg/kg Posiphen (n=9), FPI/10 mg/kg Posiphen (n=7). Significant outliers from each group were removed with the Grubb's test.



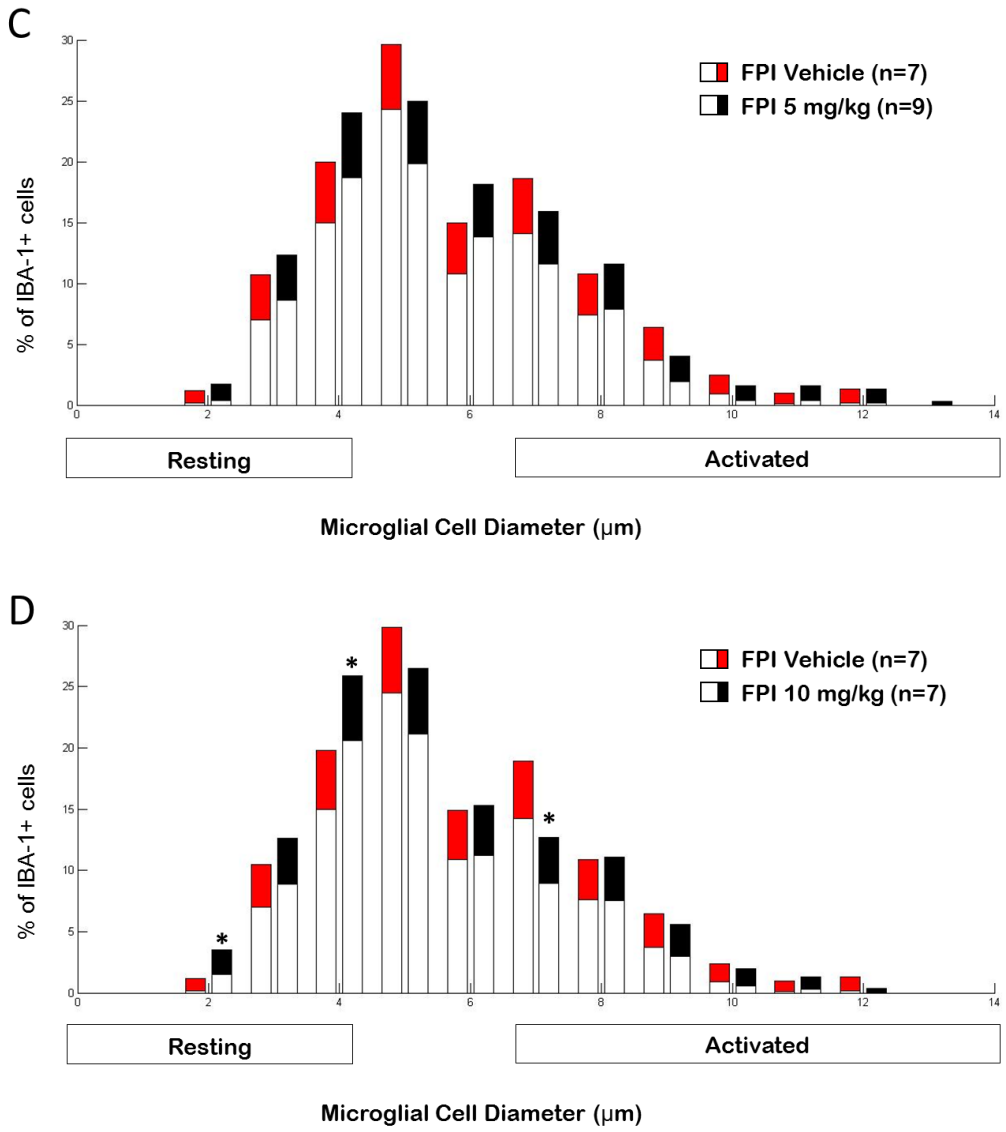


Fig. 26. **Treatment with 10 mg/kg Posiphen significantly attenuated the FPI-induced increase in microglial activation in the ipsilateral substantia nigra.** Data (Mean + 95% CI) were analyzed with Bootstrapping method, *p<0.05 (Sham FPI/vehicle n=8, FPI/vehicle n=7, FPI/2.5 mg/kg Posiphen n=9, FPI/5.0 mg/kg Posiphen n=9, FPI/10 mg/kg Posiphen n=7). **(A)** FPI rats showed a significant increase in microglial activation when compared with the vehicle control. **(B)** Treatment with 2.5 mg/kg Posiphen showed no effect on microglial activation when compared with the vehicle control. **(C)** Treatment with 5.0 mg/kg Posiphen showed no effect on microglial activation when compared with the vehicle control. **(D)** Treatment with 10.0 mg/kg Posiphen showed a slight decrease in microglial activation when compared with the vehicle control.

		Vehicle	2.5 mg/kg	5 mg/kg	10 mg/kg	PBS
# of Total Assigned Animals	CCI	12	12	15	14	
	SHAM					13
# Deaths	CCI	0	0	2	1	
	SHAM					1
# Suboptimal Surgery/Injury	CCI	2	2	2	0	
	SHAM					1
# Excluded (SA performance)	CCI	0	0	3	4	
	SHAM					1
FINAL GROUP #	CCI	10	10	8	9	
	SHAM					10

Table 3. **CCI group assignment.** A total of 66 rats were originally assigned to the study. During the course of the study period, a total of 4 rats died. After review of the surgical conditions, 7 animals were excluded from subsequent analysis (i.e., suboptimal injury with limited cortical swelling). Lastly, 8 animals were excluded from subsequent analysis based on their poor/lack of performance on the spatial alternations (SA) behavioral test. These exclusion criteria consisted of a pre-treatment SA score of less than 57% or less than 11 total arm entries (e.g., did not complete the test). Thus, the final groups for analyses were: 10 rats in the CCI/Vehicle group, 10 rats in the CCI/2.5 mg/kg Posiphen group, 8 rats in the CCI/5.0 mg/kg Posiphen group, 9 rats in the CCI/10 mg/kg Posiphen group, and 10 rats in the Sham CCI/Vehicle group.

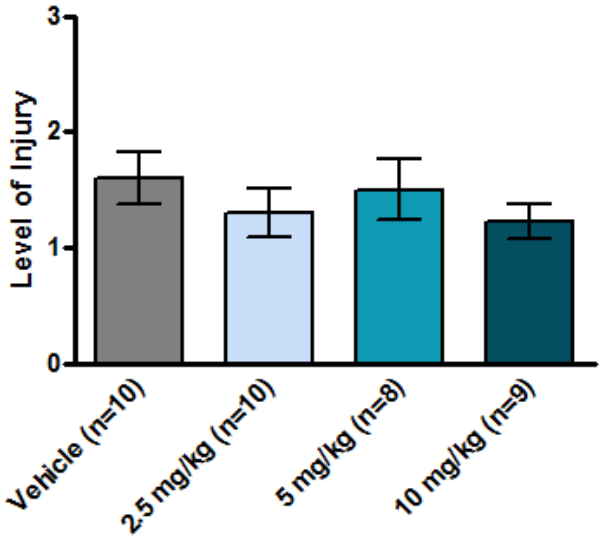


Fig. 27. **Mean level of cortical swelling for the CCI groups**. A qualitative injury scale was used to assess brain swelling: 0.5 = mild-moderate; 1 = moderate; 2 = moderate-severe; 3 = severe. Data are shown as the mean +/- S.E.M. (CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A non-parametric one-way ANOVA (Kruskal-Wallis) revealed no significant differences between groups (p=0.4930).

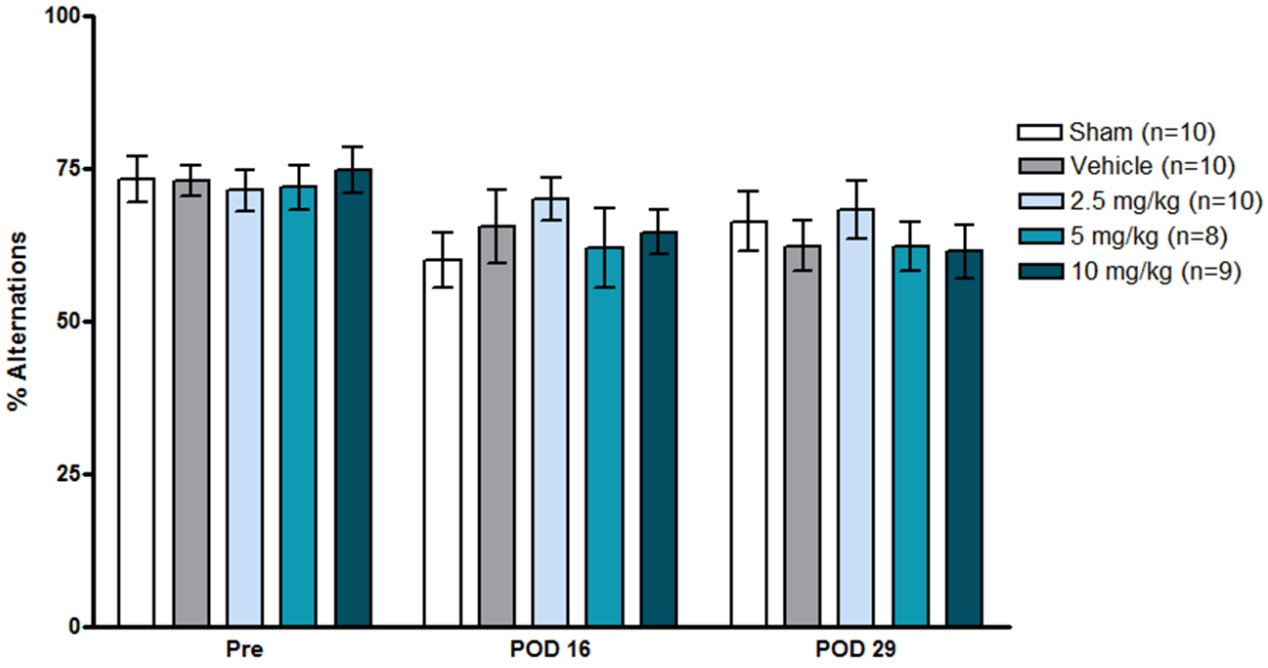


Fig. 28. **No effect of CCI injury on spatial alternations**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A two-way repeated-measures ANOVA with Bonferroni post-hoc comparisons revealed a significant effect of time ($F=5.801$, $p=0.0048$), but no significant differences between the vehicle and Posiphen-treated groups ($F=0.4714$, $p=0.7043$). Planned comparisons (sham vs. vehicle) within each time point also revealed no significant differences. POD = post-operative day.

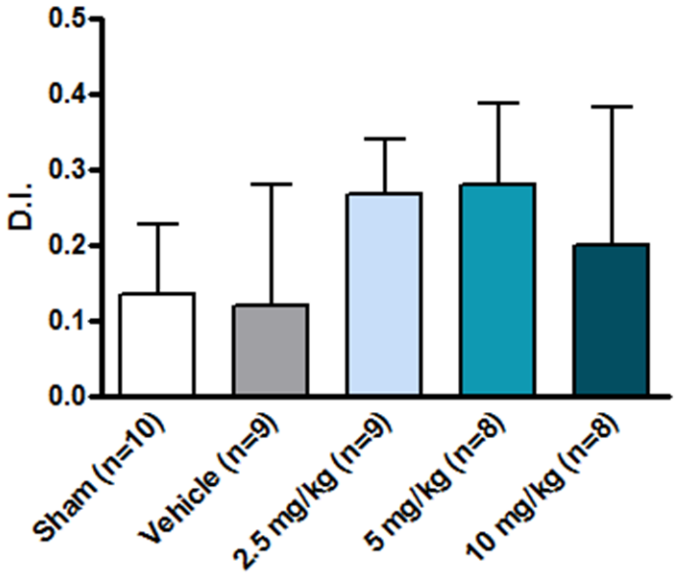


Fig. 29. **No effect of CCI injury on novel object recognition.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences ($t=0.08391$, $p=0.9341$). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups ($F=0.3039$, $p=0.8223$). Note that data from 1 animal each from the vehicle, 2.5, and 10 mg/kg groups were not used because both objects were not explored during testing. DI = discrimination index.

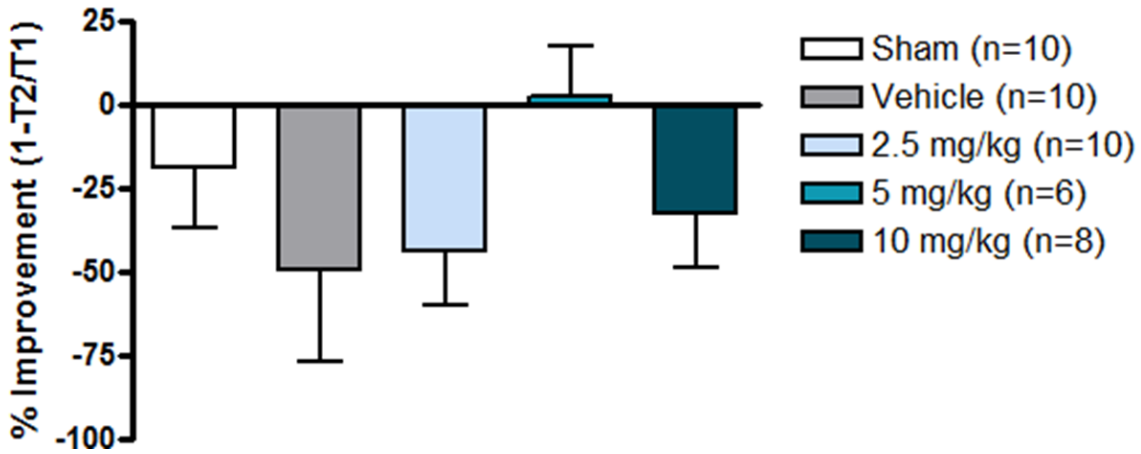


Fig. 30. **No effect of CCI injury on working memory in the Morris water maze**. Averaged data from the 3 testing days are shown as the mean +/- S.E.M (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=10, CCI /5.0 mg/kg Posiphen n=6, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences (t=0.9135, p=0.3731). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.9733, p=0.4183). Note that data from 2 animals from the 5 mg/kg group and 1 animal from the 10 mg/kg groups were not used because of a technical error during testing.

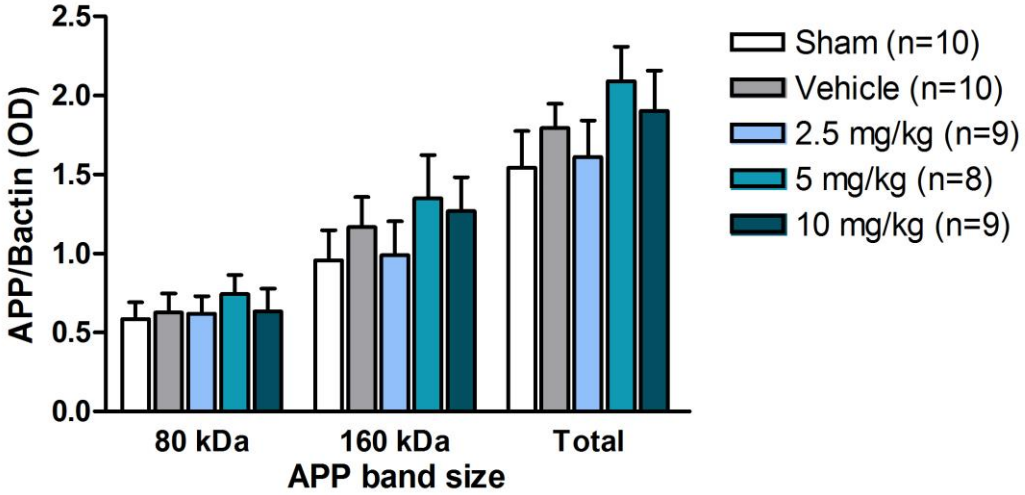


Fig. 31. **CCI injury did not significantly affect APP levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in APP (80 kDa: t=0.2434, p=0.8105; 160 kDa: t=0.7861, p=0.4420; total: t=0.8989, p=0.3806). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=0.2025, p=0.8939; 160 kDa: F=0.4745, p=0.7022; total: F=0.8299, p=0.4873). Significant outliers from each group were removed with the Grubb's test.

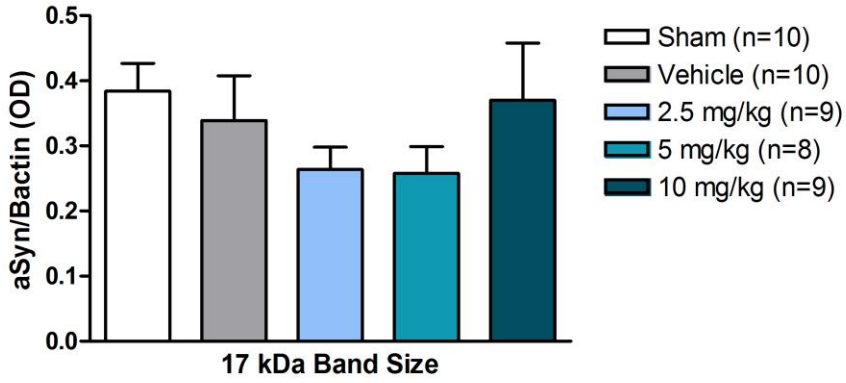


Fig. 32. **CCI injury did not significantly affect aSyn levels in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in aSyn levels (t=0.5520, p=0.5877). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.7523, p=0.5291). Significant outliers from each group were removed with the Grubb's test.

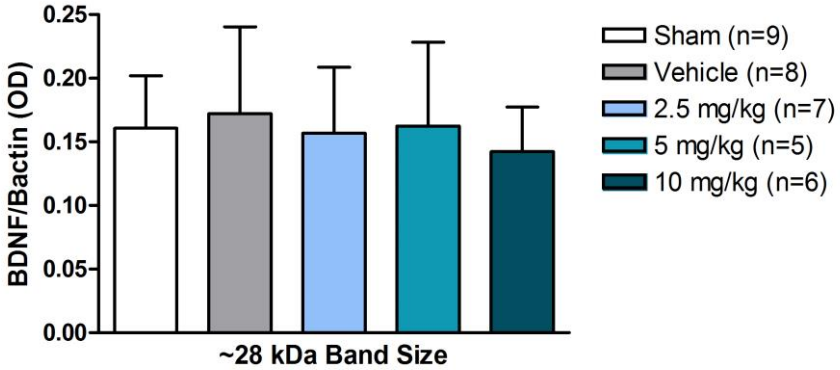


Fig. 33. **CCI injury did not significantly affect BDNF levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9, CCI/vehicle n=8, CCI/2.5 mg/kg Posiphen n=7, CCI /5.0 mg/kg Posiphen n=5, CCI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=0.1478, p=0.8845). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.0988, p=0.9599). Significant outliers from each group were removed with the Grubb's test. Note that some animals were excluded due to a technical error.

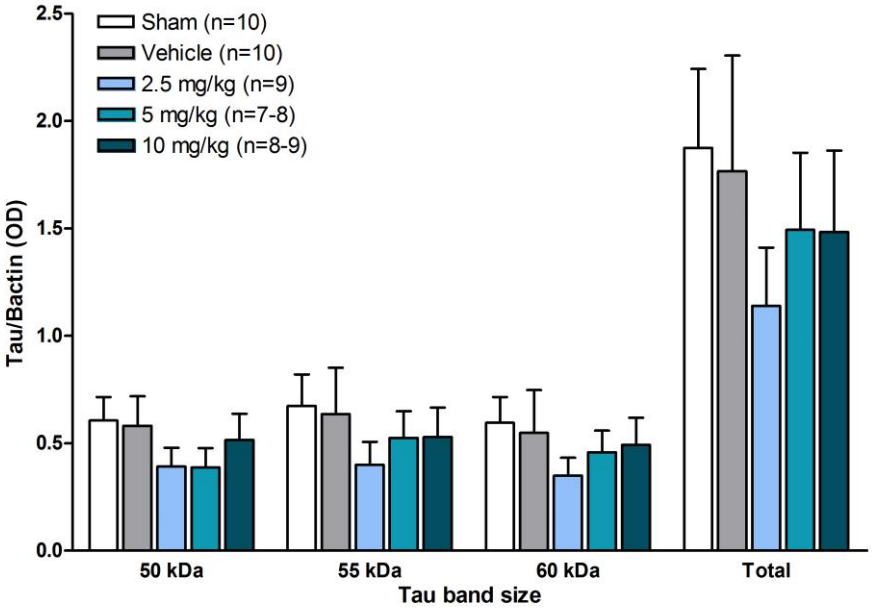


Fig. 34. **CCI injury did not significantly affect Tau levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=10, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=8-9). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=0.1404, p=0.8899; 55 kDa: t=0.1431, p=0.8878; 60 kDa: t=0.2014, p=0.8426; total: t=0.1659, p=0.8701). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.6829, p=0.5692; 55 kDa: F=0.3949, p=0.7575; 60 kDa: F=0.3721, p=0.7737; total: F=0.4062, p=0.7496). Significant outliers from each group were removed with the Grubb's test.

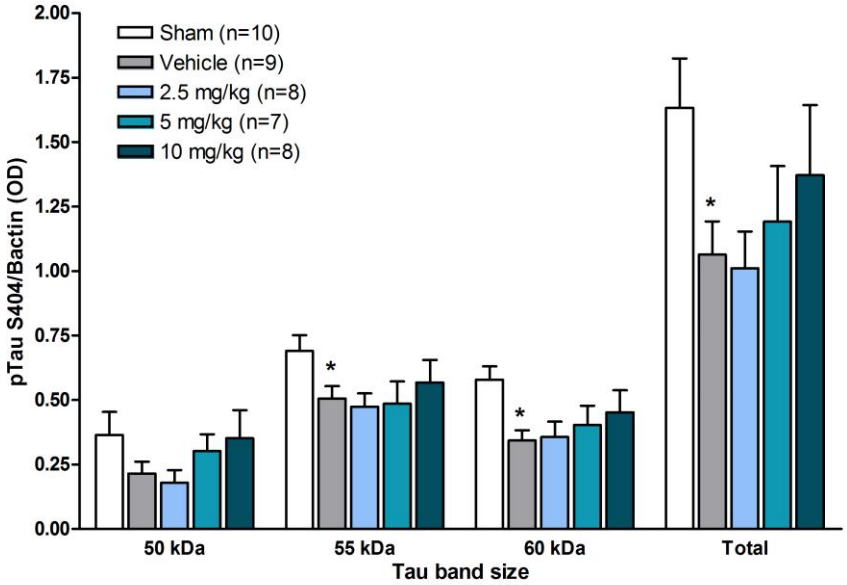


Fig. 35. **CCI injury significantly decreased pTau-S404 levels in the hippocampus**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed a significant decrease in the 55, 60 kDa, and total pS404-tau bands in injured rats (50 kDa: t=1.438, p=0.1685; 55 kDa: t=2.347, *p=0.0313; 60 kDa: t=d no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.253, p=0.3094; 55 kDa: F=0.3609, p=0.7817; 60 kDa: F=0.5898, p=0.6268; total: F=0.6937, p=0.5637). Significant outliers from each group were removed with the Grubb's test.

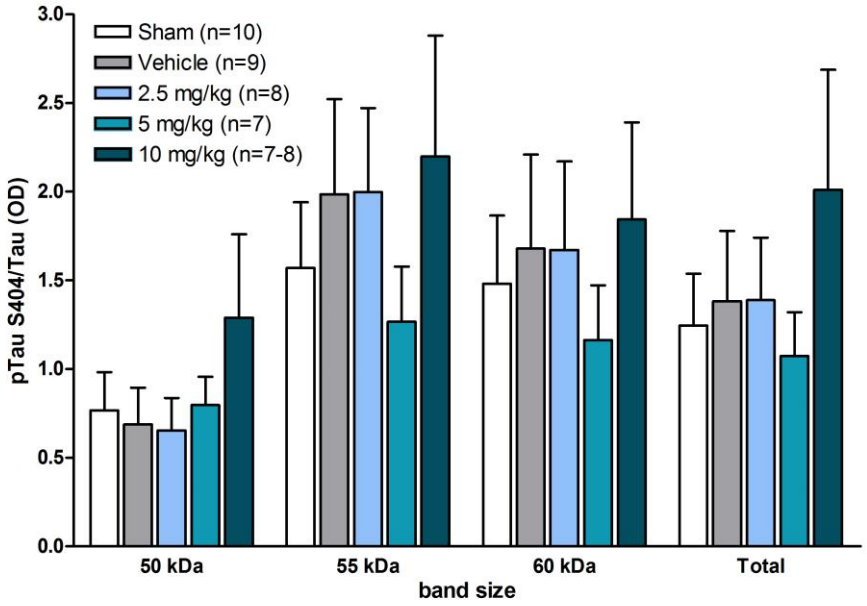


Fig. 36. **CCI injury did not significantly affect the pTau/tau ratio in the hippocampus.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7, CCI/10 mg/kg Posiphen n=7-8). A Student's t-test between sham and vehicle groups revealed no significant differences in the ratio of pTau-S404/tau (50 kDa: t=0.2692, p=0.7910; 55 kDa: t=0.6487, p=0.5252; 60 kDa: t=0.3050, p=0.7641; total: t=0.2821, p=0.7813). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=1.064, p=0.3802; 55 kDa: F=0.5384, p=0.6599; 60 kDa: F=0.3292, p=0.8042; total: F=0.7124, p=0.5528). Significant outliers from each group were removed with the Grubb's test.

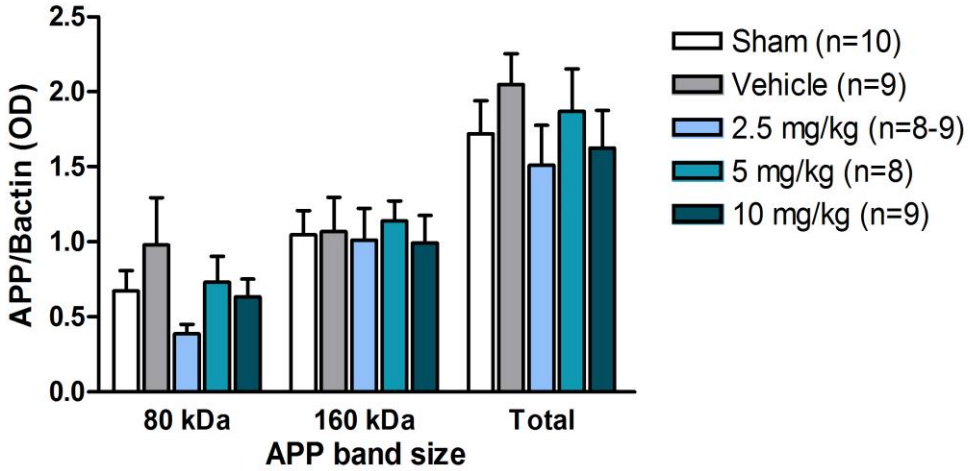


Fig. 37. **CCI injury did not significantly affect APP levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8-9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed no significant differences in APP (80 kDa: t=0.9267, p=0.3671; 160 kDa: t=0.0764, p=0.9400; total: t=1.082, p=0.2942). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (80 kDa: F=1.525, p=0.2281; 160 kDa: F=0.1097, p=0.9538; total: F=0.9482, p=0.4294). Significant outliers from each group were removed with the Grubb's test.

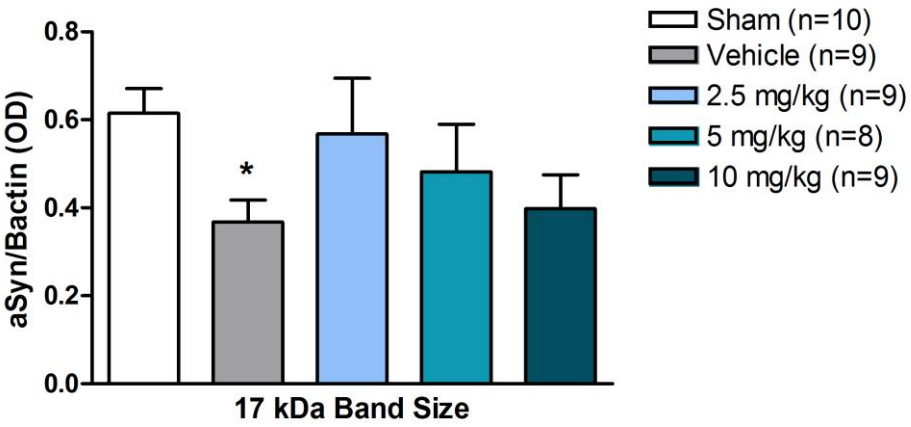


Fig. 38. **CCI injury significantly decreased aSyn levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=9). A Student's t-test between sham and vehicle groups revealed a significant decrease in aSyn levels (t=3.267, *p=0.0045). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=0.9335, p=0.4362). Significant outliers from each group were removed with the Grubb's test.

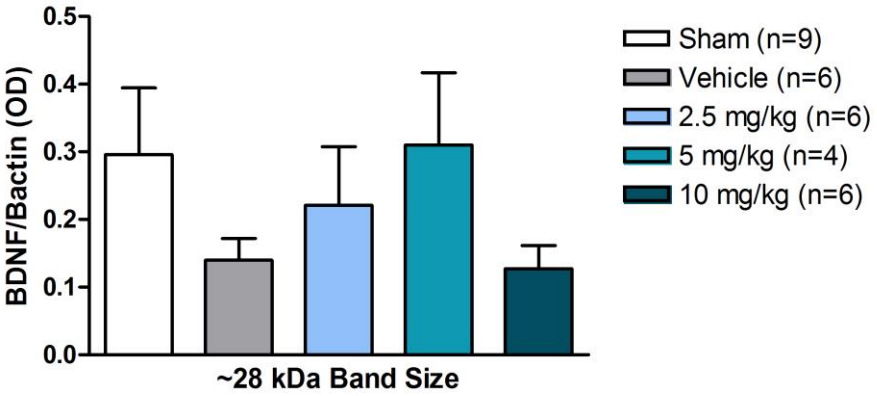


Fig. 39. **CCI injury did not significantly affect BDNF in the cortex.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9, CCI/vehicle n=6, CCI/2.5 mg/kg Posiphen n=6, CCI /5.0 mg/kg Posiphen n=4, CCI/10 mg/kg Posiphen n=6). A Student's t-test between sham and vehicle groups revealed no significant differences in BDNF levels (t=1.255, p=0.2314). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (F=1.429, p=0.2671). Significant outliers from each group were removed with the Grubb's test. Note that some animals were excluded due to a technical error.

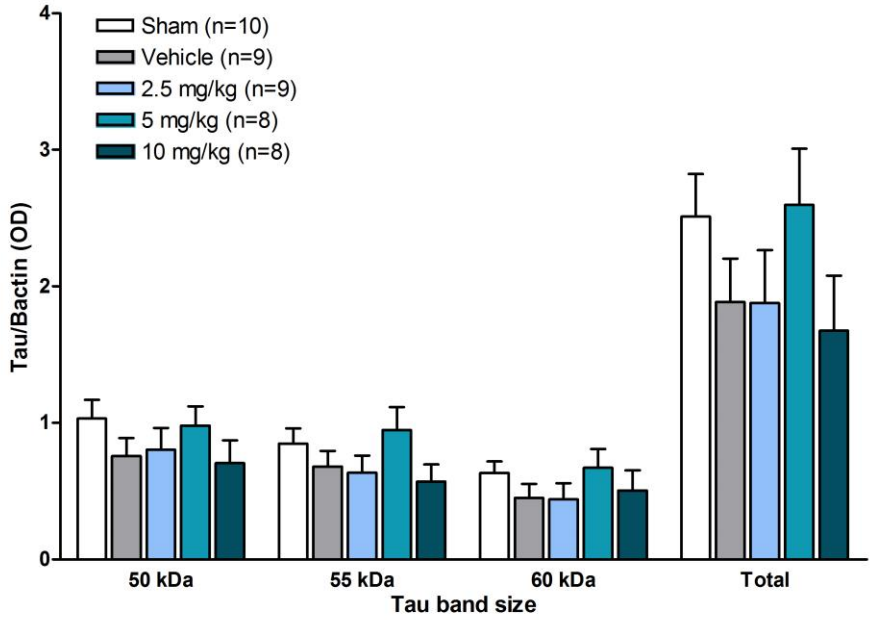


Fig. 40. **CCI injury did not significantly affect Tau levels in the cortex**. Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=9, CCI /5.0 mg/kg Posiphen n=8, CCI/10 mg/kg Posiphen n=8). A Student's t-test between sham and vehicle groups revealed no significant differences in tau levels (50 kDa: t=1.426, p=0.1721; 55 kDa: t=1.036, p=0.3147; 60 kDa: t=1.388, p=0.1830; total: t=1.401, p=0.1793). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.3204, p=0.8105; 55 kDa: F=1.452, p=0.2473; 60 kDa: F=0.6608, p=0.5824; total: F=1.071, p=0.3762). Significant outliers from each group were removed with the Grubb's test.

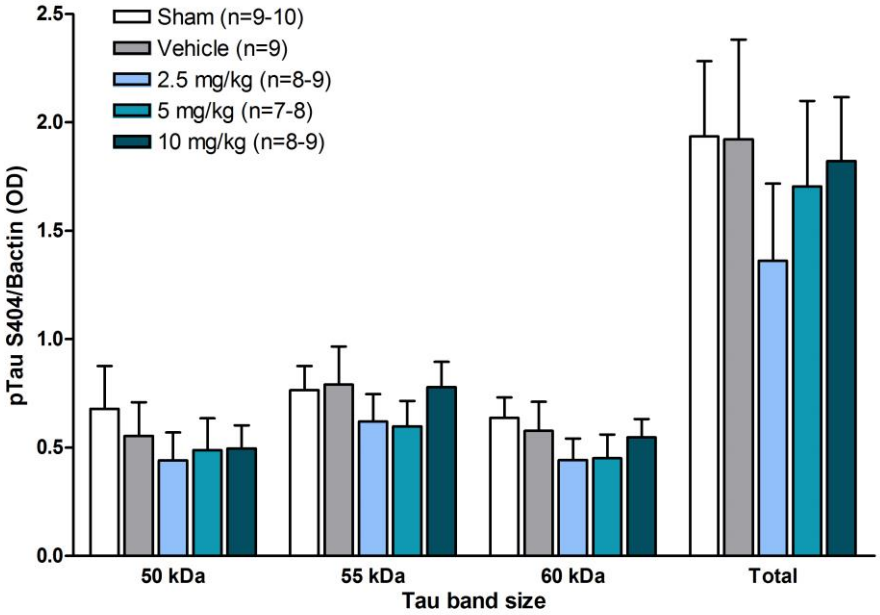


Fig. 41. **CCI injury did not significantly affect pTau-S404 levels in the cortex.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9-10, CCI/vehicle n=9, CCI/2.5 mg/kg Posiphen n=8-9, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=8-9). A Student's t-test between sham and vehicle groups revealed no significant differences in pTau-S404 levels (50 kDa: t=0.4931, p=0.6283; 55 kDa: t=0.1251, p=0.9020; 60 kDa: t=0.3674, p=0.7182; total: t=0.0236, p=0.9815). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.1179, p=0.9489; 55 kDa: F=0.5361, p=0.6613; 60 kDa: F=0.3874, p=0.7629; total: F=0.4195, p=0.7403). Significant outliers from each group were removed with the Grubb's test.

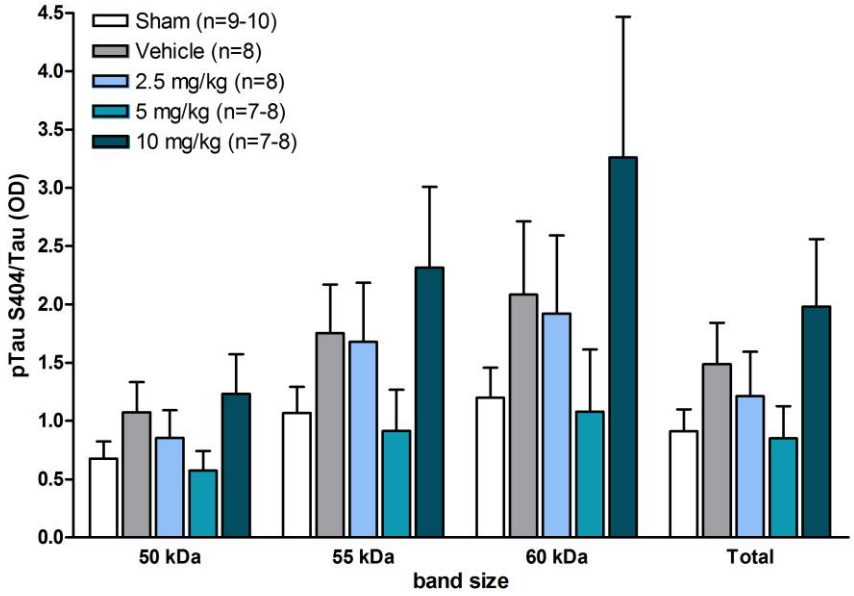


Fig. 42. **CCI injury did not significantly affect the pTau/tau ratio in the cortex.** Data are shown as the mean +/- S.E.M. (Sham CCI/vehicle n=9-10, CCI/vehicle n=8, CCI/2.5 mg/kg Posiphen n=8, CCI /5.0 mg/kg Posiphen n=7-8, CCI/10 mg/kg Posiphen n=7-8). A Student's t-test between sham and vehicle groups revealed no significant differences in the pTau-S404/tau ratio (50 kDa: t=1.406, p=0.1790; 55 kDa: t=1.496, p=0.1553; 60 kDa: t=1.359, p=0.1942; total: t=1.482, p=0.1589). A one-way ANOVA with Bonferroni post-hoc comparisons revealed no significant differences between the vehicle and Posiphen-treated groups (50 kDa: F=0.1231, p=0.3068; 55 kDa: F=1.318, p=0.2891; 60 kDa: F=1.157, p=0.3436; total: F=1.331, p=0.2840). Significant outliers from each group were removed with the Grubb's test.